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Demonstration of differences in virulence between two *Theileria parva* isolates

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ABSTRACT

In areas with a low incidence of infection due to unimodal presence of ticks, *Theileria parva* has been observed to induce a disease with relatively low pathology. This is followed by a carrier state, rather than death and therefore provides a better chance of transmission of the parasite back to the tick vector since in unimodal conditions, the different tick stages occur at different times. One isolate from such an area in Zambia, *T. parva* Chitongo, was compared for virulence with *T. parva* Muguga, isolated from an area exhibiting a continuous presence of all vector stages in East Africa. To reduce any variation due to infection dose, an *in vitro* standardized dose was used to initiate infection of groups of three local zebu cattle with each isolate. Parameters of virulence measured were prepatent period, fever, survival (based on ECF index), parasitosis, piroplasm parasitaemia and hematological parameters. Our results suggest that *T. parva* Chitongo developed a slightly later onset (1–2 days) and lower levels of parasitosis in the lymph node, causing less and later mortality. Comparison of the *in vitro* rate of transformation confirmed that the time needed to transform an infected lymphocyte took 4 days longer for *T. parva* Chitongo than *T. parva* Muguga. Elucidating the mechanism responsible for the lower virulence of *T. parva* Chitongo could be useful for designing an attenuated vaccine.

1. Introduction

Virulence is the unavoidable consequence of the competition among pathogen genotypes to exploit the host resources in order to maximize production of transmission stages (Bulls, 1994; Read, 1994). Some experimental results have predicted that competitive interactions between parasite genotypes within mixed infections should lead to more transmission stages, and consequently higher virulence, when conditions for host-transmission are optimal (Van Balen and Sabelis, 1995; Taylor et al., 2002). An unfortunate consequence of this process is the reduction in fitness of the host, because of an increased load of antigenic and pathogenic molecules from the more virulent pathogens. Evolutionary theorists have argued that pathogens will evolve to produce as much or as little virulence as is necessary to maximize transmission and this will depend on the chances of encountering the next host or vector, and the frequency of mixed or single infections.

Theileria parva is an apicomplexan intracellular protozoan parasite that infects and transforms lymphocytes of

Abbreviations: CI, confidence interval; dpi, days post-infection; ECF, East Coast fever; ED, effective dilution; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cells; PCV, packed cell volume; RBC, red blood cell; t.e., tick equivalence; WBC, white blood cells.

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cattle and African buffalo (*Syncerus caffer*). Mainly transmitted by *Rhipicephalus appendiculatus* ticks, the parasite causes a severe disease in cattle called East Coast fever, January disease or corridor disease in eastern, central and southern Africa respectively. The parasite transforms the lymphocytes to acquire a tumour-like behaviour leading to a massive lympho-proliferation of infected cells and invasion of both lymphoid and non-lymphoid organs and tissues. The clinical signs and lesions include high fever, swollen lymph nodes, and focal necrosis of lymphoid organs, lymphoma in the kidneys, hypoxia, anemia, ulcers and heamorrhages along the digestive tract, interstitial pneumonia and pulmonary oedema causing dyspnoea.

The findings of Barnett and Brocklesby (1966), Burridge et al. (1974) and Mbogo et al. (1996) suggested the presence of low virulence strains of *T. parva*. In their studies, both in field and laboratory conditions, a low virulent strain is characterized by extended incubation period, short fever duration, low mortality, low number of nuclei per schizont, low parasitemia and low average proportion of lymphocytes infected with macro and microschizonts (parasitosis) in an infected animal. However these studies have been complicated by the inability to control the dose when using ticks to initiate *in vivo* infections. Virulence has been reported to vary across isolates and cloned parasites of other theilerial species like *Theileria annulata* (Darghouth et al., 1996; Graham et al., 2001; Pipano et al., 1974; Rafyi et al., 1965).

The *T. parva* Muguga parasites circulate efficiently in a constant transmission region, where the different stages of the tick vector are present throughout the year, allowing continuous transmission of *T. parva* to and from the mammalian host. This implies that selection of higher virulence variants will not interfere much with transmission efficiency, provided infected animals only die a few days after the emergence of parasitaemia and that sick animals are exposed to immature *R. appendiculatus* ticks. In contrast, *T. parva* Katete and *T. parva* Chitongo parasites are from bimodal and unimodal (tick developmental stages occur twice or once per year) areas where the vector stages are not present throughout the year (Billiouw et al., 2002; Mulumba et al., 2001) and, thus, the adults and nymph or nymph and larvae or larvae and adults do not occur together frequently. In such situations, a carrier state in the bovine host is necessary to guarantee transmission from infecting to infectable tick stages. In this case, higher virulence and the resulting early death of the host will interfere with the successful transmission of the parasite. Hence the expected presence in those areas of parasite genotypes associated with lower parasite loads, lower disease severity and more chronic infections (Marcotty et al., 2002). However, it has been argued that the disease severity following primary infection is largely dependent on the infective sporozoite dose (Radley et al., 1974; Samantaray et al., 1980; Preston et al., 1992). High doses, inoculated naturally by ticks or in the form of tick extracts (called stabilates when cryopreserved), invariably kill all susceptible hosts before they can mount a protective response. Lower doses produce more variable results, from lethal to subclinical reactions or absence of infection. Quantitation of the infective material prior to any virulence study is therefore essential. The introduction of an *in vitro* titration model (Marcotty et al., 2004) in which an effective dose (ED) was determined and taken as the unit of the initial infective sporozoite dose concentration has improved quality assessment and quantitation in the sporozoites production process.

The objective of this study was to compare the virulence of an isolate from a constant transmission region, *T. parva* Muguga, with the virulence of an isolate from a low transmission region, *T. parva* Chitongo, as defined by their ability to induce disease *in vivo* and their efficacy to infect and transform bovine target cells *in vitro*. The assessment of clinical reactions was based on prepatent period, onset and duration of fever, parasitosis (% schizont-infected lymphocytes), parasitemia (% piroplasm-infected erythrocytes), blood cell counts and time to death.

2. Materials and methods

2.1. Theileria stabilates

The Muguga stabilate (TpM 3087 DSG 4221) was prepared from dissected salivary glands. It was derived after 4 passages through cattle and ticks from 'Muguga stabilate 10', that itself had been transmitted an unknown number of times since it had been isolated in Kabete, Kenya (Brown et al., 1977; Morzaria et al., 1995). The *T. parva* Chitongo stabilate (TpC CA0401) was prepared from whole ground-up ticks as described by Mbao et al. (2005), and had been passaged three times since pick-up from the field.

2.2. In vitro sporozoite titration

An infective sporozoite can be defined as one that can infect a cell and develop into a schizont. The objective of this experiment was to estimate the stabilate dilutions of the two isolates that provide equivalent numbers of infective sporozoites. This was done by carrying out *in vitro* infections and comparing the stabilate dilutions that produced the same number of wells positive for at least one schizont-infected cell (effective dilution).

Target peripheral blood mononuclear cells (PBMC) were collected from donor zebu cattle (*Bos indicus*) free of ECF and maintained under tick-proof conditions. *T. parva* tick-derived stabilates were titrated according to the *in vitro* method of Marcotty et al. (2004). Both stabilates were diluted in a series of 12 twofold dilutions, as shown in Table 1. The mixing of sporozoites and cells, incubation and the scoring of the schizont-positive wells at 10 dpi were done as described in Marcotty et al. (2004).

The binary data (96 observations for each stabilate) were analysed using a logistic regression in Stata9/SE (StataCorp, 2006) and the effective dilution which would result in 50% of the wells being infected (the ED50) (Marcotty et al., 2004) was calculated. The proportion of positive wells was the response variable and the logarithm of the stabilate dilution and the isolates titrated were explanatory variables. The ln(ED50) difference between the two stabilates was calculated using the non-linear combination of estimators tool in Stata and transformed in an effective dose ratio.

Table 1
Top (Muguga) and bottom (Chitongo) showing the sporozoites stabilate dilution, the scores of positive wells obtained for each dilution and the calculated average proportion of schizont-infected wells after Stata analysis.

Dilution	Scores	Average
10	8/8	0.99997
20	8/8	0.999905
40	8/8	0.999703
80	8/8	0.999068
160	8/8	0.997085
320	8/8	0.990916
640	8/8	0.972058
1280	6/8	0.917323
2560	6/8	0.779668
5120	6/8	0.530201
10,240	3/8	0.264672
20,480	0/8	0.102974
Dilution	Scores	Average
10	8/8	0.999645
20	8/8	0.998888
40	8/8	0.996522
80	8/8	0.989175
160	8/8	0.966824
320	7/8	0.902859
640	6/8	0.747746
1280	3/8	0.485965
2560	3/8	0.231665
5120	0/8	0.087726
10,240	0/8	0.029757
20,480	0/8	0.009687

2.3. Comparison of in vitro transformation

This experiment compared *in vitro* transformation and cell growth between the two isolates, using equivalent doses of infective sporozoites. The isolates were compared at different sporozoite titres. Transformation was monitored by following the percentage of schizont-infected cells over time.

Sporozoite stabilates were serially diluted in complete RPMI 1640 ECF medium (12 twofold dilutions) and incubated with total peripheral blood lymphocytes as described above (Marcotty et al., 2004), but a total volume of 200 μl instead of 50 μl of the sporozoites suspensions were used and incubated with pelleted PBMC (3×10^6 cells) for 1.5 h with shaking every 15 min to initiate infection. The infected cells were washed once in culture medium and finally resuspended in 2 ml of RPMI 1640 ECF medium into a 24-well plate. The rest of the procedure was completed as described above. The experiment was carried out in duplicate.

Sampling was done daily starting at day 1 up to 12 dpi. Cells were resuspended by pipetting up and down and 200 μl of the cells suspension was taken from each well and subjected to cyto-centrifugation and Giemsa staining. The proportion of schizont-infected PBMC was calculated by scoring the differential counts of total PBMC against the schizont-positive PBMC from counting a minimum of 400 cells per slide under the microscope at 1000-fold magnification, making sure all cells in every field were counted.

The data was also analysed using a linear regression in Stata. The square root of the proportion of infected cells was arcsin-transformed to assure normality (Osborne, 2002). Explanatory variables included the stabilate (binary: Chitongo or Muguga), the day post-infection (discrete), the interaction between the latter two and the logarithm of the stabilate dose (continuous). The validity of the model was evaluated by plotting the residual quantiles against the quantiles of a normal distribution in a *Q–Q* plot.

2.4. Comparison of virulence in vivo

This experiment attempted to compare parasite and host infection parameters after challenge with a similar number of infective sporozoites. A total of six *T. parva*-free cattle (from local zebu breed, *Bos indicus*) were selected for the experiment. The animals were divided in two groups of three animals. One group was inoculated subcutaneously above the ear with 1 ml of 1/20 diluted *T. parva* Muguga and the other group with 2 ml of 1/10 diluted *T. parva* Chitongo (using the same stabilate batches as above). At these stabilate dilutions, samples contained an equivalent number of infective sporozoites as measured by the *in vitro* infection assay. The animals were monitored daily for rectal temperature, parasitosis (percentage of lymphocytes carrying macroschizonts), parasitemia (piroplasms for 1000 erythrocytes). The number of granulocytes and lymphocytes per volume, as well as total white blood cell count, hemoglobin level, packed cell volume (PCV), red blood cell count (RBC) were monitored at 5, 7, 12, 14, 16, 19 and 21 dpi, with a haematology cell counter MS4 Vet (Melet Schloesing Laboratoires, France).

Detection and estimation of the parasitosis were carried out on sub-parotid and prescapular lymph node needle biopsy smears from all the infected cattle each day throughout the course of the infection. The degree of parasitosis was scored daily on a scale from 0 to 3 (Rowlands et al., 2000). For clinical analysis and euthanasia, the animals were monitored independently by a veterinarian who did not have knowledge on the grouping of animals. A decision to euthanize an animal because of ethical concerns, was made by the veterinarian and depended on the following symptoms: the animal was too weak to stand, had high fever of over 40.0 °C over 5 days, showed lethargy and was off feed for more than 24 h, showed palour of mucous membranes, had a persistent dry cough for more than 3 days, had laboured breathing, had mucoid discharges from the nose and muzzle and had a staggering gait with a stooping head. If at least five of these symptoms appeared together with parasitological signs, then the animal was euthanized (Rowlands et al., 2000). The experimental design and the use of animals were approved by the ILRI Animal Care & Use Committee (IACUC) and animals were treated in accordance with its guidelines.

Rectal temperatures, parasitosis, white blood cells and the survival time were compared between the two stabilates in Stata9/SE (STATA9, 2006). For rectal temperature, the data was split into 3 time groups: a prepatent period from day 0 to 4, a fever rising period from day 5 to 9 and a patent period from day 10 to the first death (Rowlands et al., 2000). The data from the prepatent and

patent periods were analysed in a linear latent and mixed model using individual animals as random effect and the groups (infected with Chitongo or Muguga), the periods (0–4 days pi and 10–14 days pi) and the interaction between them as discrete explanatory variables. Although, there is no data available for the pre-infection period, the limited variability observed in the prepatent period validate the successive measurement-being an intrinsic demonstration of limited variability among animals.

Similarly, ln(WBC/ml) was compared statistically on days 5, 7 and 12 in a linear latent and mixed model. Analyses of the time to score level 3 parasitosis in local lymph node and the time to euthanasia were done using a lognormal survival model and the stock as only explanatory variable. Lognormal models better fit non-monotonic hazard rates (STATA9, 2006) (i.e. the hazard rates are very low on the first dpi, reach a maximum 1–2 weeks pi, after which they decrease to low levels again).

3. Results

3.1.1. Equivalent doses of T. parva stabilates

The average proportion of schizont-infected wells for each dilution series and for each parasite strain was obtained in Stata (Table 1 and depicted as a function of dilution factor as the unit (Fig. 1) and used to determine the effective dose ratio at 95% confidence interval. The dilution ratio of the stabilate doses of Muguga and Chitongo that provided an equivalent proportion of schizont-infected wells was 4.9, with 95% CI of 2.6–8.9 (Fig. 1). A dilution ratio of fourfold for Muguga versus Chitongo was used in all subsequent experiments to provide equivalent sporozoite infection doses, and while this suggests a 25% higher dose for Muguga than Chitongo, it is within the 95% confidence margin.

3.2. Comparison of in vitro infections

The proportion of infected cells increased with time post-infection and this increase was significantly different for both stabilates ($P < 0.01$). Several dilutions pairs of Muguga and Chitongo, with equivalent infective doses, were compared. Estimates for two of these equivalent dilutions are represented in Fig. 2 with their 95% confidence intervals.

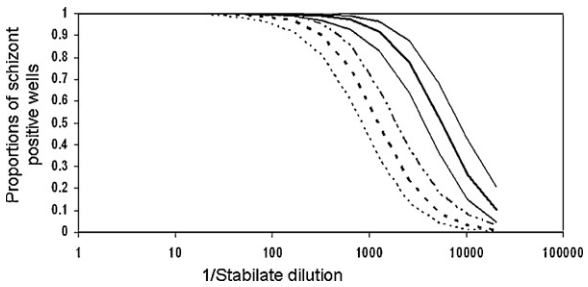

Fig. 1. Logistic curve of the *in vitro* titration test . The bold, continuous line represents the Muguga doses, while the bold, dotted lines show the Chitongo doses. The thin lines show the 95% confidence intervals for each stabilate.

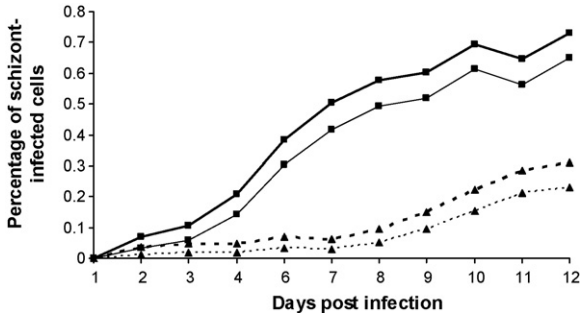

Fig. 2. Comparison of *in vitro* transformation with equivalent sporozoite doses of *T. parva* Chitongo (1/20) (thick, triangles, broken line) and Muguga (1/80) (bold, squares, full line). The graph shows the percentage of schizont-infected cells per well over time, no positive cells were found on day 1. The thin lines show fourfold dilutions of the initial ones, Chitongo (1/80) (thin, triangles, broken lines) and Muguga (1/320) (thin, squares, full line). The other dilutions are not shown here, but were included in the statistical analysis.

Each graph could be divided into three phases: a initial lag phase, that corresponded to the time needed for the infective sporozoite stage to mature into a schizont and transform its host cell, a second phase recognized by a rapid increase in the percentage of schizont-positive cells in the well and the final phase when cell growth was limiting, probably due to medium conditions. The time needed for the Muguga sporozoites to infect, mature and transform was the same for the two dilutions represented in Fig. 2: approximately 3 days. After that time, parasitized cells grew and expanded rapidly, although slightly slower in the diluted sample ($P < 0.001$). The same three phases were observed with the Chitongo strain. A significant difference with the Muguga strain was observed in the percentage of schizont-infected cells between dpi 4 and 11 ($P < 0.001$).

From Fig. 2 it is clear that the Chitongo sporozoites took a longer time to mature and transform their host cell than the Muguga sporozoites, about 7 days instead of three. Furthermore, dilution of sporozoites reduced the abundance of schizont-infected cells at any given day. As shown in the log phase growth in Fig. 2, a fourfold dilution (1/320 as final dilution) resulted in a delay of about a day for the abundance of infected cells to reach the same level as in cultures initiated with less diluted stabilates (1/80). This was observed both with Muguga and Chitongo stabilates. However, the stabilate effect is far more pronounced than the dose effect. To account for the difference observed between the two stabilates on day 8 by dose alone, a 2581-fold dilution of the Muguga stabilate would be necessary (the 95% CI, calculated using a non-linear combination of parameters based on the delta method, are 852–7815).

3.3. Comparison of in vivo infection parameters

A number of infection parameters were monitored over time in two groups of three cattle infected with equivalent doses of sporozoites from both *Theileria* strains.

3.3.1. Temperature profile

Body temperatures averaged by stock and day from day 1 post-infection up to the day of euthanasia or termination

of the experiment and their standard deviations were plotted graphically against the days post-infection (Fig. 3). Generally Muguga-infected animals exhibited a higher peak temperature than Chitongo-infected animals. Statistically, the peak fever (between day 9 and 14) induced by Muguga is significantly higher than Chitongo's ($P = 0.028$; with a mean temperature of 41.1 compared to 40.7 for Muguga and Chitongo respectively). While all Muguga-infected animals had died by day 16, the temperature profile from day 15 onward showed decreasing trends in all Chitongo-infected animals.

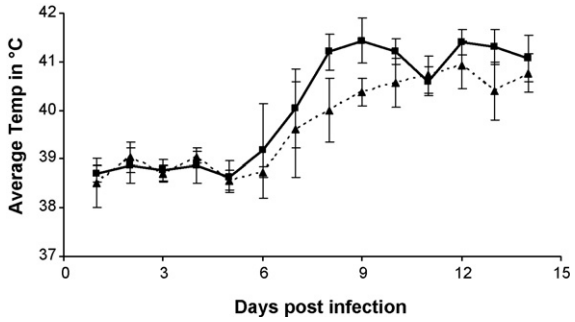

Fig. 3. Average body temperatures for Chitongo (thin broken line, triangle) and Muguga (bold line, boxes) infected animals and standard deviations by day post-infection.

3.3.2. Time to euthanasia

The median survival time for Muguga (15 days) was significantly shorter than for Chitongo (22 days) ($P < 0.001$). One of the *T. parva* Muguga infected animals was euthanized on dpi 14, another on dpi 15 before samples could be collected and one on dpi 16; the Chitongo-infected animals were euthanized on days 19 and 22 dpi, while the third one recovered from infection.

3.3.3. Parasitosis

Schizonts appeared in the lymph node at the same time for both *Theileria* strains (day 6 pi). However, severe parasitosis occurred earlier in the *T. parva* Muguga-infected animals ($P = 0.002$) jumping straight from level 1 to level 3 (Fig. 4) at least in all the animals within the group. In contrast, parasitosis in *T. parva* infected animals went through level 2 except for BA034 and in the later stages the animals started clearing the schizont-infected cells 15 days pi (Fig. 4, graphs right column). The three *T. parva* Muguga-infected animals reached level 3 parasitosis on days 10, 10 and 11 respectively, while the *T. parva* Chitongo animals reached level 3 on days 11, 12 and 13 respectively, suggesting a 1–2 days difference. No parasite clearance was observed in the Muguga-infected animals since their schizont levels remained high until euthanasia.

3.3.4. Parasitaemia

Piroplasms appeared at low levels (<0.1%; not visible on graph) in the blood circulation at the same time (day 11) in both infection groups and no significant difference was observed between the two groups (Fig. 5).

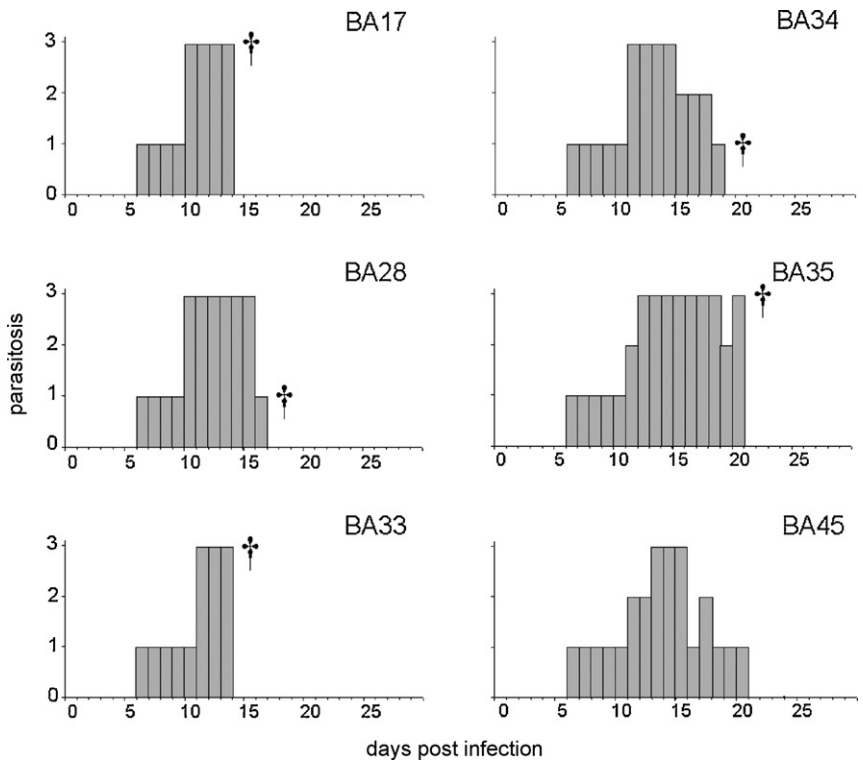

Fig. 4. Histogram showing the parasitosis levels measured daily in lymph node; the first column shows the parasitosis levels in Muguga-infected cattle (BA17, BA28 and BA33) and the second column Chitongo-infected cattle (BA34, BA35 and BA45).

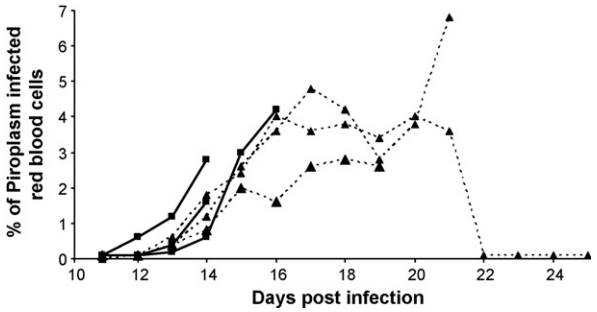

Fig. 5. Graph showing piroplasm parasitaemia. The thick continuous lines with square symbols show the Muguga-infected animals whereas the thin broken lines with triangle symbols show the Chitongo-infected animals.

3.3.5. Haematology parameters

At day 5 and 7 the *T. parva* Muguga-infected animals had significantly ($P < 0.001$; $P < 0.05$) higher white blood cell counts (Fig. 6A) compared to *T. parva* Chitongo-infected animals. Unfortunately, we do not have pre-infection data, and therefore do not know whether this was as a consequence of the infection or because of a pre-infection condition. They also had a higher number of granulocytes (Fig. 6B). White blood cells counts decreased by day 12 and 14, but significantly more so in Muguga-infected animals than in Chitongo-infected animals ($P < 0.001$) (Fig. 6A). Again this correlated with similar changes in granulocyte numbers (Fig. 6B). When granulocytes were represented as a percentage of the white blood cells, the difference between Muguga and Chitongo on day 14 is even more apparent (Fig. 6C). By 14 dpi (0, 1 and 2 days before euthanasia, respectively), the Muguga-infected cattle were all agranulocytic. The Chitongo-infected cattle survived this critical period, and their WBC and granulocyte counts increased after that time.

No evident differences were observed in other haematological parameters (RBC, haemoglobin and PCV) between the two stabilates before the first animal was euthanized.

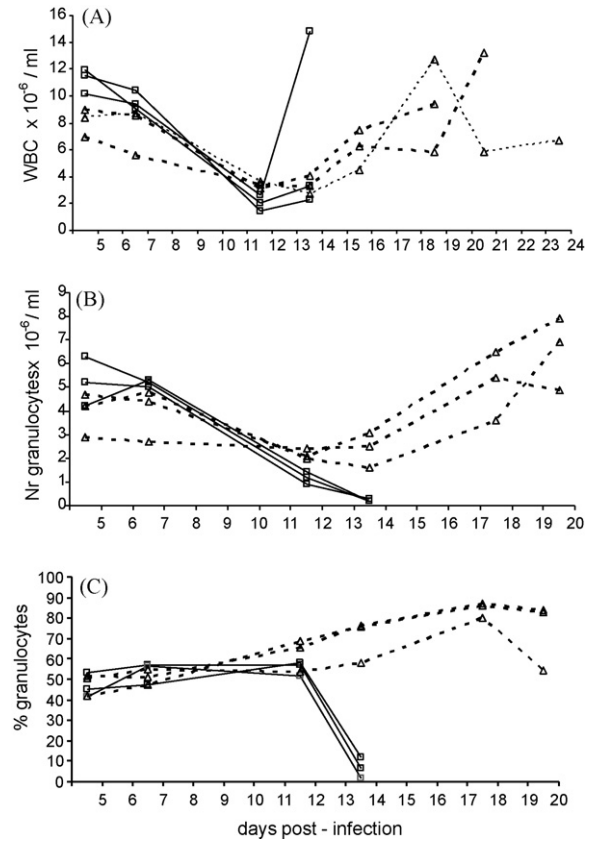

Fig. 6. Graphs showing the white blood cells count (A), granulocyte numbers (B), and the proportion of granulocytes as a percentage of the total WBC (C). Dotted line with triangles represent *T. parva* Chitongo-infected animals, full line with squares represent *T. parva* Muguga-infected animals.

4. Discussion

In this study we quantified the infective dose in two *T. parva* sporozoite stabilates to provide for a quantitative comparison of viable infective sporozoites giving equal dose. Sporozoite quantitation based on *in vitro* titration (Marcotty et al., 2004, Mbao et al., 2005), revealed that the Muguga stabilate had about five times more infective sporozoites than the Chitongo stabilate. Differences in storage conditions or treatments after freezing can affect viability or the number of infective sporozoites per sample; however, we assume that this will not change the virulence of a parasite, which is an intrinsic and genetically determined characteristic. Since subsequent experiments used vials from the same Chitongo and Muguga stabilates and since always two vials from each batch were mixed before infection, we assume that storage conditions and variability between samples were not the cause of the differences observed between the two stabilates.

When comparing the virulence of the two isolates *in vitro*, after infecting PBMC with similar doses of infective sporozoites, the major difference that was observed was the longer time that *T. parva* Chitongo-infected cells needed to start multiplying and reach a logarithmic growth phase, compared to the *T. parva* Muguga-infected cells. This could be explained in two ways. The first is that Chitongo sporozoites were less efficient to mature into schizonts after infection and therefore took a longer time to transform their host cell. The other is that fewer cells got infected by Chitongo sporozoites or fewer cells developed mature schizonts, resulting in a longer time for the transformed cells to reach their logarithmic growth phase. This observation could be the most significant reason for Chitongo's lower virulence, as survival of the animal depends on a competition between the rate of multiplication of infected cells and the development of protective responses.

Chitongo-infected animals reached their peak of parasitosis 1–2 days after Muguga-infected animals. This correlates with the delay observed for *T. parva* Chitongo to reach maximal growth *in vitro* and suggests that this delay could be the main cause for its lower virulence. A low initial number of infected cells might allow the host more time to induce an effective protective response before infected cells reach pathological levels. Conversely the rapid multiplication of Muguga before a protective

response is in force, might lead to high parasite loads, severe pathology and early death.

Several other *in vivo* parameters show that the Muguga infection was progressing faster and causing more pathology. Temperatures in the Muguga-infected animals were statistically higher between 9 and 14 dpi, in Muguga than Chitongo infected animals. Concerning the hematological parameters, the steep decreases in WBC, granulocyte and lymphocyte counts between 5 and 12 dpi could be explained either by sequestration of these cells from blood and homing into tissues (Wilson et al., 2002; Shiels et al., 2006) or because of a block in haemopoietic activity and failure in production of new cells. The slightly higher numbers of WBC at 5 and 7 dpi in Muguga-infected cattle compared to Chitongo-infected cattle, could be a pre-infection condition (we unfortunately do not have these data) or is a consequence of the higher parasitosis and a higher number of stimulated lymphoblasts in Muguga-infected animals. Similarly, the higher number of granulocytes might be due to a release from the marginal pool in response to stronger, systemic inflammatory conditions in Muguga-infected animals. Granulocytes also decreased in blood, probably as a consequence of inflammatory conditions caused by infected lymphocytes in the tissues. Between 12 and 14 dpi we observed a major difference between Muguga and Chitongo-infected cattle. In the Muguga-infected animals, granulocyte numbers decreased dramatically in blood (Fig. 6B) and their percentage in the WBC dropped to very low levels (Fig. 6C). This agranulocytic condition in the Muguga-infected animals (Fig. 6B) occurred just before the animals died. While a block in haemopoietic activity could explain a rapid decline in granulocyte numbers (they have a short half life of 2 days; Edwards et al., 2003), it seems more likely that the cells were migrating to the tissues attracted by inflammatory mediators released by accumulation of infected lymphocytes. This may cause massive disruption in life-critical tissues, such as lungs or kidneys, and contribute to severe pathology in the animal. The WBC and granulocyte counts in Chitongo-infected cattle also decreased around the critical period between 12 and 14 dpi, but to a much lesser extent and cell numbers in the blood recovered afterwards.

All these factors mentioned above indicate a lower virulence of Chitongo than Muguga. A longer transformation period is one contributing factor. Another factor could be a difference in the pathogenicity of cells infected by Chitongo and Muguga, respectively. Comparing animals infected with autologous cells transformed *in vitro* by different Theileria isolates might give an answer, although it is not known how closely such cells resemble *in vivo*-infected cells. Such a study has been carried out, and demonstrated significant differences in virulence among *T. parva* isolates (Morzaria et al., 1995). Unfortunately, in this study the phenotypes of the *in vitro*-infected cells were not known. It has been shown that host cell phenotype can also affect the ensuing pathology (Moreau et al., 1999; Morrison et al., 1996).

Finally, it could be argued that the difference in virulence may be related to the multiple passages of *T. parva* Muguga through cattle and ticks, which may have selected a more virulent strain, while the low virulence of the *T. parva* Chitongo stabilate may be related to its recent isolation and low number of passages. It has been shown that isolates can be composed of several genotypes and recombination and selection may occur during each passage through the tick (Katzer et al., 2006). The *T. parva* Chitongo stabilate we used in this experiment has been passaged three times through cattle since it was isolated, while the *T. parva* Muguga stabilate has been passaged considerably more (see Section 2.1). Further comparative experiments, using quantified doses of fresh isolates and perhaps cloned parasites would be needed to confirm this hypothesis.

In conclusion, an equivalent infective dose of *T. parva* Chitongo sporozoites induced less pathology and less severe disease in local zebu cattle compared to *T. parva* Muguga. The probable cause of *T. parva* Chitongo's lower virulence may be that it needs a longer time to reach logarithmic growth phase, either because it takes a longer time to mature into a schizont and transform its host cell, or because less host cells become transformed. Knowledge of this mechanism could be helpful in designing attenuated vaccine strains.

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