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Comparison of the virulence of *Trypanosoma congolense* strains isolated from cattle in a trypanosomiasis endemic area of eastern Zambia

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Abstract

The virulence of 31 genetically different *Trypanosoma congolense* strains belonging to the Savannah subgroup and isolated from cattle at 11 sites in a trypanosomiasis endemic area of eastern Zambia was compared. Virulence testing, done in OF1 mice, revealed three virulence categories. Strains were considered extremely virulent when the median survival time ranged between 5 and 9 days. Moderately virulent strains had a median survival time between 10 and 30 days and low virulence, more than 30 days. For each strain, the prepatent period was determined and the PCV of the infected animals was measured at regular intervals. A total of six (19.4%) strains belonged to the extremely virulent category with a short prepatent period (mean 2.3 ± 0.3 days), high parasitaemia, decline in PCV of $15.6 \pm 1.1\%$ during the first 7 days p.i. and a short median survival time (mean 6 days). The remainder of the strains belonged to the moderate (13 strains) or low (12 strains) virulence categories with median survival times of 13 and 60 days, respectively. They had longer prepatent periods (means 3.2 ± 1.6 days and 3.5 ± 1.6 days for moderately virulent and strains with low virulence, respectively) and the decline in PCV was less steep (decline of 14.2 ± 0.6 and $9.7 \pm 0.6\%$ during the first 7 days of infection with moderately virulent strains and strains with low virulence, respectively). Extremely virulent strains were isolated from cattle at four sampling sites with 60% of the cattle from one sampling site harbouring such extremely virulent strains. Results from this study demonstrated substantial differences in the virulence of *T. congolense* strains of the Savannah subgroup, isolated in one geographic area from a single host species. On the assumption that information on virulence obtained from tests in mice can be extrapolated to cattle, the high proportion of strains with low to moderate virulence is thought to be attributed to the important role of susceptible cattle as reservoirs of trypanosomes in the study area and the ensuing selection against extremely virulent strains.

Keywords: *Trypanosoma congolense*; Savannah subgroup; Virulence; Bovine trypanosomiasis; Epidemiology; Zambia

1. Introduction

Tsetse-transmitted livestock trypanosomiasis is a major constraint to livestock production in 37 countries of sub-Saharan Africa (Swallow, 2000). In livestock, *Trypanosoma congolense*, *Trypanosoma vivax* and, to a lesser extent, *Trypanosoma brucei brucei* are the causal agents. An infection with one of those trypanosome species may result in a chronic, debilitating, emaciating and often fatal disease but the outcome of the infection differs substantially between trypanosome, between livestock species and within a livestock species among breeds (Connor and Van den Bossche, 2004). In cattle, *T. congolense* (subgenus *Nannomonas*) is considered the most pathogenic trypanosome species. Based on the molecular markers *T. congolense* has been divided into four subgroups, i.e. Savannah, Forest, Kilifi and Tsavo (Hide and Tait, 2004). Limited experiments comparing the virulence of one strain of each subgroup in mice and cattle have shown differences between the subgroups with the *T. congolense* strain of the Savannah subgroup being the most virulent (Bengaly et al., 2002a,b).

Differences in the expression of disease have been found in different *T. congolense* Savannah foci with the disease having little impact in some areas compared with other areas (Van den Bossche, 2001). There is a suggestion that even within a

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subgroup, virulence may differ between strains. To confirm this, the virulence of several genetically different *T. congolense* strains isolated from cattle reared in a trypanosomiasis endemic area of eastern Zambia was compared in mice.

2. Materials and methods

2.1. Study area and isolation of trypanosomes

Trypanosoma congolense was isolated from communal cattle reared in a trypanosomiasis endemic area and sampled at 11 sampling sites (Fig. 1) located in the Katete and Mambwe Districts of eastern Zambia (Machila et al., 2001). The area is highly cultivated with a cattle population of approximately 8–10 animals/km^2 (based on an aerial survey conducted in August 1997). Large game animals are absent. *Glossina morsitans morsitans*, which takes the majority (75%) of its bloodmeals on cattle (Van den Bossche and Staak, 1997), is the only tsetse species present and cattle constitutes the main reservoir of trypanosomes. Trypanosomes were isolated in mice. Briefly, for each infected bovine, a volume of 0.5 ml was injected i.p. into two OF1 mice. The parasitaemia of the injected mice was checked three times a week by direct examination of tail blood. Parasitaemic mice were euthanised and the blood collected was used for stabilate preparation. A total of 31 genetically different strains of *T. congolense*, characterised using a modified Amplified Fragment Length Polymorphism (AFLP) technique (Masumu, unpublished data), were used in this study. They all belonged to the Savannah subgroup (Geysen et al., 2003).

2.2. Virulence testing

The virulence of 31 strains was compared in OF1 mice. All the strains were at their fifth or sixth passages in mice. Before infection, each of the strains was expanded into two OF1 mice. Wet tail-blood films of the infected mice were examined microscopically at 2-day intervals to estimate the parasitaemia (Herbert and Lumsden, 1976). When the parasitaemia reached $10^{7.8}$ trypanosomes/ml, tail-blood was extracted and diluted into Phosphate buffer Saline Glucose (PSG) to achieve a concentration of 10^5 parasites in a total volume of 0.2 ml. This volume was injected i.p. in six OF1 mice for each strain. A group of six mice, injected i.p. with 0.2 ml of PSG was used as control.

For each strain, the prepatent period (day of the first appearance of parasites in the blood), the level of parasitaemia, the survival rate and the development of anaemia were recorded. Mortality in infected and control mice was recorded daily. The parasitaemia was estimated, daily during the first 2 weeks and thereafter every 2 days, using wet tail-blood film method described by Herbert and Lumsden (1976). An animal was considered parasitologically negative when no trypanosomes were detected in at least 50 microscopic fields. The PCV of tail-blood was measured using the micro-centrifugation method. It was measured before infection, every 2 days for the first 2 weeks p.i., and once a week for the remainder of the experiment (up to 90 days p.i.). Animal ethics approval for the experiment infections was obtained from the Ethics Commission of the Institute of Tropical Medicine, Antwerp, Belgium (Ref DG001-PD-M-TT).

Trypanosome strains were divided in different virulence categories depending on the median survival time of infected mice. Strains were considered extremely virulent when the median survival time was short and ranged between 0 and 9 days. The moderately virulent strains had a median survival time between 10 and 30 days and strains with low virulence had a median survival time of more than 30 days.

2.3. Statistical analysis

The statistical analyses were carried out using Stata 8.0 software (StataCorp, 2003). A linear regression was used to

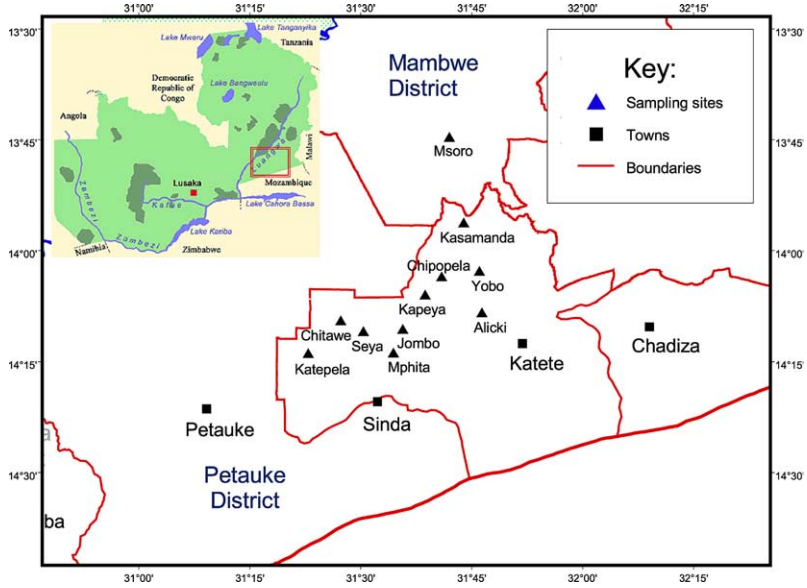

Fig. 1. Location of sampling sites in the Katete and Mambwe Districts of eastern Zambia.

analyse prepatent periods in function of the virulence categories. The PCV values measured on surviving mice on days 0 and 7 were analysed in a cross-sectional linear regression model. To ensure normality of the response variable, the square root of the PCV values were arcsin transformed (Osborne, 2002). The virulence category, the day p.i. and the interaction between them were used as categorical explanatory variables whereas the effect of strain was taken as a random effect. Finally, the non-linear combination of estimators function (Oehlert, 1992) was used to calculate the statistics related to the decline in PCV.

3. Results

Based on the median survival time, six strains (19.4%) were classified as extremely virulent, 13 strains (41.9%) were considered moderately virulent and the remainder 12 strains (38.7%) had low virulence in mice. The Kaplan–Meier survival curves for each virulence category are presented in Fig. 2. Throughout the 90 days observation period no mortality was recorded in the control group.

All infected mice developed parasitaemia. Independently of the virulence category, infection resulted in a steep increase in the parasitaemia. In the mice infected with *T. congolense* strains belonging to the low or moderate virulence categories the average parasitaemia stabilised at a level varying around $10^{7.5}$ and $10^{8.1}$ parasites/ml blood from day 8 p.i. onwards. In mice infected with extremely virulent strains, on the other hand, the parasitaemia continued to increase until death (Fig. 3). The prepatent period was significantly shorter in *T. congolense* strains belonging to the extremely virulent category compared to strains of the moderately virulent category and those belonging to low virulence category ($P=0.015$ and <0.001, respectively) (Table 1). All infected mice developed anaemia soon after the onset of parasitaemia (Table 1).

During the first 7 days p.i., the average PCV in individual mice decreased from 19 to 33% depending on the virulence category of the *T. congolense* strain. The decline was steepest

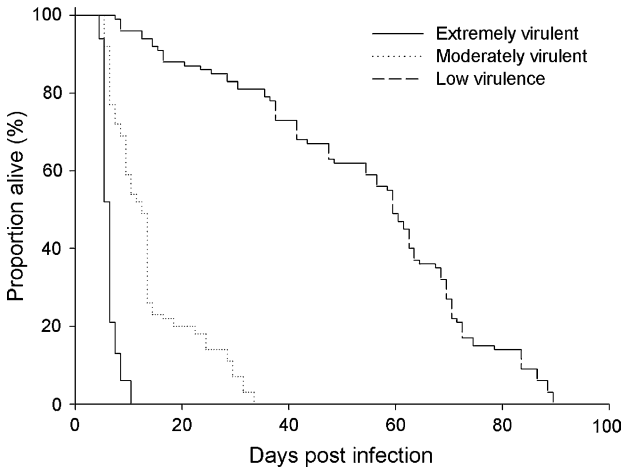

Fig. 2. Kaplan–Meier survival curves of *Trypanosoma congolense* strains belonging to the extreme, moderate and low virulence category.

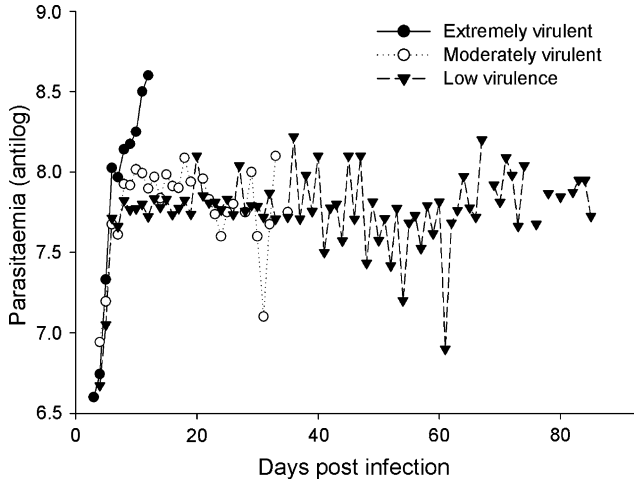

Fig. 3. Average parasitaemia of *Trypanosoma congolense* strains belonging to the extreme, moderate and low virulence category on different days p.i. The prepatent period was significantly shorter in extremely virulent strains compared with strains of the moderate or low virulence category ($P=0.015$ and <0.001, respectively).

Table 1
Median survival time, average prepatent period and average decline in PCV on day 7 of *Trypanosoma congolense* strains belonging to the extreme, moderate or low virulence category

Parameters	Virulence category		
	Extreme	Moderate	Low
Median survival time (in days)	6	13	60
Average prepatent period (in days)	2.3±0.3	3.2±1.6	3.5±1.6
Average decline in PCV on day 7 (in absolute values)	15.6±1.1	14.2±0.6	9.7±0.6

in mice infected with the extremely virulent strains followed by mice infected with strains of the moderate and low virulence categories (Fig. 4). Statistical analysis showed a significant difference in the PCV on day 7 p.i. between *T. congolense*

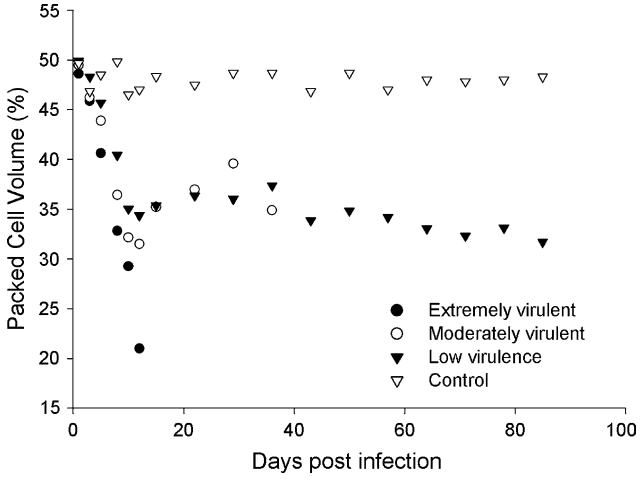

Fig. 4. Average packed cell volume of *Trypanosoma congolense* strains belonging to the extreme, moderate and low virulence category on different days p.i. The difference in PCV between extremely or moderately virulent strains and strains with low virulence, on day 7 p.i., was statistically significant ($P<0.001$).

Table 2
Number of *Trypanosoma congolense* strains, isolated at one of the 11 sampling sites, belonging to each of the three virulence categories

Sampling site	Number of strains	Number of strains in each virulence category		
		Extreme	Moderate	Low
Alicki	5	3	1	1
Chitawe	5	0	4	1
Chipopela	4	0	2	2
Kasamanda	3	1	1	1
Seya	3	0	0	3
Yobo	3	1	1	1
Kapeya	2	0	2	0
Mphita	2	0	1	1
Msoro	2	0	1	1
Jombo	1	0	0	1
Katepela	1	1	0	0

strains belonging to the extreme and moderate virulence categories compared with strains with low virulence ($P < 0.001$). In mice infected with moderately virulent or strains with low virulence, the average PCV stabilised but remained low from day 10 p.i. onwards. However, in mice infected with extremely virulent strains, the PCV continued to decline until death.

Extremely virulent strains were isolated from cattle at four of the 11 sampling sites. With the exception of strains from Alicki or Katepela, the majority of isolates from cattle at the other sampling sites belonged to the moderate or low virulence categories (Table 2). Only one strain was isolated at Katepela. In Alicki, on the other hand, extremely virulent strains constituted 60% of the isolates. This suggests an uneven distribution of virulent *T. congolense* strains.

4. Discussion

Results of the virulence testing in mice showed unequivocally that genetically different *T. congolense* strains belonging to the same genetic subgroup and isolated in one geographic area from one host species may differ substantially in their virulence. Since the growth rate of such cloned trypanosome strains is a relatively stable trait (Postan et al., 1986; Diffley et al., 1987) the observed differences in virulence could not be attributed to the serial passages of the strains in mice.

Field isolation through inoculation in mice may result in partial selection of strains that are well adapted to development in rodents. Hence, the range of strains and their distribution in the three virulence categories may not be a true reflection of the field population. Unfortunately, unbiased techniques for isolating trypanosomes in sufficient numbers are not yet available.

Notwithstanding the shortcomings inherent to the methodology, results from the infections clearly show that the virulence of *T. congolense* strains of the Savannah subgroup may differ as much as the virulence between strains of the Savannah and Kilifi or Forest subgroup (Bengaly et al., 2002 a, b). Indeed, 12 out of 31 strains that were compared showed a particularly long survival time in mice similar to the clones belonging to the Kilifi and Forest subgroup (Bengaly et al., 2002a). In addition, the observed differences in the development of parasitaemia and the anemia of strains of the Savannah subgroup but belonging to the different virulence categories resemble those observed by Bengaly et al. (2002a) between strains belonging to the different subgroups. Since the findings of Bengaly et al. (2002a,b)were based on the observations made on one single clone of the Kilifi and the Forest subgroups it is likely that as considerable variation in virulence exists between clones of each of those subgroups as among clones of the Savannah subgroup. Hence, before drawing firm conclusions on differences in virulence between subgroups, further investigations comparing more clones of the Kilifi and Forest subgroups are required.

Extrapolating the results obtained in mice to cattle requires caution. However, experiments conducted by Bengaly et al. (2002a,b) have shown good concordance between results of virulence tests in mice and tests of the same trypanosome strains in cattle. On the assumption that the results obtained in mice do represent the strain's characteristics in cattle, the question remains which factors contribute to the relative prevalence of virulent and less virulent strains in the cattle population of a particular area? As with the clones of *Trypanosoma cruzi* (Tibayrenc et al., 1986), it can be hypothesised that the heterogeneity in the characteristics reflects adaptation to different transmission cycles.

This hypothesis is supported by the observation that in trypanosomiasis endemic areas where susceptible cattle breeds constitute the main host of tsetse and are the reservoir of trypanosomes (domestic transmission cycle), the impact of the infection on production is generally low (Van den Bossche, 2001). This was attributed to the fact that strains most likely to survive in a susceptible host population should have low virulence resulting in a mild disease. In the study area from which 31 strains were obtained, cattle constitutes the main host of tsetse and constitutes the main reservoir of trypanosomes. Such a situation may favour the persistence of trypanosome strains that cause such mild infections. This seems to be the case in the study area where only 19.4% of all strains were extremely virulent. Furthermore, extremely virulent strains were isolated from cattle from four of the 11 sampling sites and 50% of extremely virulent strains were isolated from cattle at one sampling site (Alicki) suggesting an uneven spatial distribution of such virulent *T. congolense* strains and possibly also an uneven spatial distribution of disease impacts. Despite the presence of extremely virulent strains in the trypanosome population, the impact of the disease on cattle production in the study area is generally low (Doran, 2000). This is in accordance with the observed high proportion of strains with low or moderate virulence. However, further research is required to characterise possible focal differences in disease impact (i.e. at Alicki) or in mechanism that may reduce the impact of infections with extremely virulent strains in cattle.

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