FIG 1: Cytomorphological differences between intact (A) and akinetoplastic (B) blood stream forms of *T congolense*. Bar 11 µm

FIG 2: Dividing forms of *T congolense*, showing a partly intact form (arrow head) and an AK form with a single kinetoplast (arrow). Bar 11µm

Cytomorphological differences between AK forms and intact forms are shown in Fig 1. In highly resistant strains, several dividing AK forms with a single kinetoplast (Fig 2) were also observed within four to 10 hours after treatment with a sublethal dose of both diminazene aceturate and isometamidium chloride. All dividing forms were therefore excluded from the AK counts.

No parasites were seen in either the BR or BSS groups 24 hours after treatment with 28·0 or 45·0 mg kg^{-1} diminazene aceturate. The most consistent and significant differences ($P < 0.01$) were seen 10 hours after treatment. Comparisons of AK rates at each dose level 10 hours after treatment are summarised in Table 2. AK rates of less than 30 per cent corresponded to no effect of the drug on the infection. AK rates of more than 30 per cent but less than 50 per cent corresponded to a temporary clearance of infection followed by relapsing parasitaemia less than 14 days after treatment. All AK rates of more than 50 per cent corresponded to a permanent cure of infections, with no relapses observed during the 60-day observation period. All treatment levels, with the exception of 3·5 mg kg^{-1} diminazene aceturate, resulted in the permanent cure of the BSS strain, with AK rates above 50 per cent and as much as 85 per cent at the 45·0 mg kg^{-1} dose level. In contrast, only the 45 mg kg^{-1} diminazene aceturate treatment resulted in a permanent cure of the BR-clone. This clone had AK rates of less than 30 per cent when treated with 3·5 mg kg^{-1} diminazene aceturate; more than 50 per cent at 45 mg kg^{-1} and between 30 and 50 per cent for all other relapsing treatment levels, that is, 7, 14 or 28 mg kg^{-1} doses.

TABLE 2: Responses of the BR and BSS strains to various diminazene aceturate treatments based on parasitaemia, dose efficacy and 10 hour AK rates (experiment 1)

Group	Dose (mg kg^{-1})	Avg parasitaemia (h)* 0	4	10	24	48	Dose efficacy†	AK rate (%) ± SD‡
BR	3·5	6+	6+	6+	6+	4+	0/3 NE	28·0 ± 1·1**
BSS	3·5	5+	4+	3+	2+	NP	0/3 TE	36·1 ± 1·9
BR	7·0	6+	5+	5+	3+	NP	0/3 TE	34·5 ± 2·1**
BSS	7·0	5+	4+	3+	1+	NP	3/3 PE	51·1 ± 1·8
BR	14·0	5+	5+	4+	3+	1+	0/3 TE	38·9 ± 1·1**
BSS	14·0	5+	4+	2+	NP	NP	3/3 PE	57·0 ± 1·0
BR	28·0	5+	4+	3+	NP	NP	0/3 TE	37·3 ± 1·9**
BSS	28·0	5+	4+	3+	NP	NP	3/3 PE	80·0 ± 5·0
BR	45·0	5+	4+	3+	NP	NP	3/3 PE	54·0 ± 3·0**
BSS	45·0	5+	3+	1+	NP	NP	3/3 PE	85·0 ± 1·5

(n = 3 at all levels)
*Average parasitaemia
NP No parasite seen in the peripheral blood
†The number of mice permanently cured/the number treated
NE No effect, TE Temporary effect, PE Permanent effect
‡Mean ± standard deviation (n = 3)
**Values of AK induction were significantly different between the BR and BSS strains at $P < 0.01$

Similar observations of AK induction were also found in the isometamidium chloride treatment groups, with considerably lower AK rates in the SR than the BSS strain (Table 3). The only dose which effected a permanent cure of the SR clone was 16 mg kg^{-1} isometamidium chloride, which induced a 10 hour AK rate of 53 per cent. All treatment levels given to the BSS strain resulted in the curing of the infection while the 10 hour AK rates in each BSS group were over 50 per cent. No parasites were detected 24 hours after treatment with 8 or 16 mg kg^{-1} isometamidium chloride. Significantly higher ($P < 0.01$) 10 hour AK rates were observed in the BSS-strain than in the SR clone at all treatment levels.

Significantly different AK-induction rates ($P < 0.01$) were also observed between both the BR or

TABLE 3: Responses of the SR and BSS strains to various isometamidium chloride treatments based on parasitaemia, dose efficacy and 10 hour AK values (experiment 1)

Group	Dose (mg kg⁻¹)	Avg parasitaemia (h)* 0	4	10	24	48	Dose efficacy†	AK rate (%) ± SD‡
SR	0·5	5+	5+	5+	5+	6+	0/3 NE	8·3 ± 1·3**
BSS	0·5	5+	4+	3+	2+	NP	3/3 PE	54·3 ± 2·0
SR	1·0	5+	5+	5+	5+	4+	0/3 NE	16·8 ± 2·2**
BSS	1·0	5+	4+	3+	2+	NP	3/3 PE	53·8 ± 1·4
SR	2·0	5+	5+	6+	5+	4+	0/3 NE	29·0 ± 2·7**
BSS	2·0	5+	4+	2+	1+	NP	3/3 PE	52·6 ± 1·3
SR	4·0	5+	4+	4+	3+	2+	0/3 TE	43·0 ± 2·2**
BSS	4·0	6+	5+	4+	2+	NP	3/3 PE	52·7 ± 2·5
SR	8·0	5+	5+	4+	3+	NP	0/3 TE	39·7 ± 2·5**
BSS	8·0	5+	4+	3+	1+	NP	3/3 PE	61·8 ± 1·5
SR	16·0	5+	5+	4+	2+	NP	3/3 PE	53·5 ± 1·6**
BSS	16·0	5+	3+	2+	NP	NP	3/3 PE	65·0 ± 6·1

*Average parasitaemia (n = 3 at all levels)
NP No parasite seen in the peripheral blood
†The number of mice permanently cured/the number treated
NE No effect, TE Temporary effect, PE Permanent effect
‡Mean ± standard deviation (n = 3)
**Values of AK induction were significantly different between the SR and BSS strains at $P < 0.01$

TABLE 4: Responses of the four original trypanosome isolates to diminazene aceturate (DA) or isometamidium chloride (IC) treatments based on parasitaemia, dose efficacy and 10 hour AK rates (experiment 2)

Group	Treatment	Dose (mg kg⁻¹)	Avg parasitaemia* 0 h	24 h	Dose efficacy†	AK-rate (%) ± SD‡
CP 1	DA	7·0	(3)5+	(3)NP	0/3 TE	35·4 ± 1·2
2	IC	1·0	(3)5+	(3)4+	0/3 NE	9·9 ± 0·9
KW 1	DA	7·0	(3)5+	(3)NP	0/3 TE	45·0 ± 1·9
2	IC	1·0	(3)5+	(3)5+	0/3 NE	8·3 ± 1·6
CS 1	DA	7·0	(3)5+	(3)NP	3/3 PE	53·2 ± 1·8
2	IC	1·0	(3)5+	(3)NP	3/3 PE	55·2 ± 3·6
MW 1	DA	7·0	(3)5+	(3)6+	0/3 TE	33·1 ± 2·9
2	IC	1·0	(3)5+	(3)5+	0/3 NE	13·2 ± 2·9

*Average parasitaemia (n = 3 at all levels)
NP No parasites seen in the peripheral blood
†Number of mice permanently cured/number treated
NE No effect, TE Temporary effect, PE Permanent effect
‡Mean ± standard deviation (n = 3)

SR clones and the BSS-strains following pararosaniline treatments (data not shown). Both the BR and SR clones had AK rates of approximately 4 per cent while the rate in the BSS strains was approximately 14 per cent. Although there was no clearance of parasitaemia in these groups, the observed AK rates were significantly different 10 hours and 24 hours after treatment despite being generally lower than those obtained for diminazene aceturate or isometamidium chloride.

Results from experiment 2 also revealed a similar pattern of AK-induction rates to those given above (Table 4). All 10 hour AK rates of more than 50 per cent corresponded to permanently effective doses; ineffective doses had values below 30 per cent while relapsing cases had AK rates between 30 and 50 per cent. In both the diminazene aceturate and isometamidium chloride treatment groups, the AK-induction results, for each strain, showed a positive correlation with known drug sensitivities in mice.

Discussion

Owing to an apparent increase in reports of trypanocide resistance in sub-Saharan Africa in recent years (Tacher 1982, Trail et al 1985, Braide 1987), and a lack of novel trypanocidal compounds in development (Trail et al 1985), it is possible that drug resistance may become a major problem in the treatment of trypanosomiasis in the future. However, the incidence of trypanocide-resistance is difficult to determine as little if any regular monitoring of drug efficacy is carried out on the few trypanocides now available for use against animal trypanosomiasis. Therefore, the prevalence of drug-resistant trypanosomes in the field still remains unclear. A major obstacle to widespread screening for resistance is the lack of suitable methods for the detection of resistant field isolates.

The induction of cytomorphological changes as an indicator for estimating drug efficacy and the degree of resistance of *T b gambiense* in infected mice is well established (Inoki and Matsushiro 1959, Ono and Inoki 1973, Ono and Nakabayashi 1978). AK induction is a phenomenon associated with drugs whose modes of action have been described as the inhibition of kDNA synthesis, thus arresting parasite replication (Inoki 1956, Ono 1977).

Compounds known to induce AK bloodstream forms include diminazene aceturate, hydroxystilbamidine, acriflavine, pararosaniline, ethidium bromide and furazolidone (Werbitzki 1910, Inoki 1956, Sakamoto 1963, Mühlpfordt 1963, Delain et al 1971, Ono and Inoki 1973, 1975, Riou and Benard 1980). In the present study,

isometamidium chloride was found to be a very strong inducer of AK bloodstream forms in both *T congolense* and *T b brucei* strains. It is possible that the mode of action of isometamidium chloride, in addition to that of diminazene aceturate (Braide 1987), involves the inhibition of kDNA synthesis. The high AK rates observed following treatment with high diminazene aceturate doses may be attributed to a possible double mode of action: inhibition of the kDNA and, or, a non-specific pathogenic effect of the drug on susceptible strains as described following hydroxystilbamidine treatment (Ono 1977). Although pararosaniline was not as efficient as diminazene aceturate or isometamidium chloride in inducing AK forms, or in the clearance of parasitaemia, it could be used to distinguish between resistant and susceptible trypanosome strains to these two drugs.

It was evident from the results presented here that variations in the AK-induction responses exist between all trypanosome clones and populations used, both diminazene aceturate and isometamidium chloride were very effective in inducing AK forms in susceptible trypanosomes. Although it has previously been reported that cattle curative doses do not always correspond to results obtained from drug assays performed in mice (Sones et al 1988), it was, however, of interest to note that both diminazene aceturate and isometamidium chloride standard cattle doses induced AK forms in mice to a level which enabled a distinction to be made between susceptible and resistant trypanosomes. While no quantitative analysis was carried out on the numerical proportions of the *T b brucei* and *T congolense* species, and on the resultant AK forms in the mixed infections of the original KW and MB stocks, both species were considered in the AK counts. The observed AK-induction phenomenon, however, applied to both species.

In conclusion, therefore, it may be possible to estimate the efficacy of diminazene aceturate or isometamidium chloride against strains or cloned populations of both *T congolense* and *T b brucei* using the determination of AK-induction rates by drug pressure. It may also be possible to establish the degree of resistance of trypanosomes in vivo, although very low levels of resistance may not be detected. Further verification of the applicability of this technique in cattle is recommended.

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Trypanosoma congolense: The Use Of 4,6-Diamidino-2-Phenylindole (DAPI) in the Akinetoplastic Induction Sensitivity Test

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African animal trypanosomiasis control is being seriously affected by the emergence of trypanosome strains resistant to almost all the commonly used trypanocides [5, 19, 21]. Under the field conditions, the incidence of such strains is difficult to assess using the present drug screening methods which normally use mice and/or *in vitro* assays. Results obtained from the conventional use of mouse infection and treatment method may not correspond with the actual curative doses for cattle [18], and an observation period of more than 30 days is necessary to confirm curative doses. While, *T. vivax* which is not infective to rodents can not be screened using mice. The present quantitative assays which use *in vitro* radioisotope or growth inhibition techniques, are also inappropriate for wide-scale application because of the high expertise and the complicated logistics involved in distinguishing drug-resistant from drug-susceptible strains following incubation in the presence of drugs [9, 16].

African trypanosome has a kinetoplast which contains genetic material, kDNA [12–15]. Certain compounds including diminazene aceturate (Berenil, Hoechst) or isometamidium chloride (Samorin, May and Baker) are known to induce akinetoplastic trypomastigotes, Ak-forms [3, 8, 13, 15]. The rate of the Ak-form induction in trypanosomes following drug exposure has an inverse correlation with the sensitivities to the drug. The more resistant the strain is to the drug, the lower the Ak-rate is. The more susceptible the strain is to the drug, the higher the rate is. Thus, drug sensitivities in African trypanosomes can easily be established using the Ak-rates [3, 8]. In this study, we tried to determine whether 4,6-diamidino-2-phenylindole (DAPI), a synthetic trypanocide which readily binds DNA to give rise to a fluorescent DNA-DAPI complex [10], could be of any staining value for rapid detection of the Ak-form.

Three clonal populations of *Trypanosoma congolense*, derived from two field isolates found in Zambia [2] were used. The cloning was carried out according to the established methods [11]. The Berenil-resistant (BR) clone was derived from the protozoon isolated in Chipata and it had minimal curative dose (MCD) values of 45 mg/kg Berenil and 1 mg/kg Samorin. The Berenil-Samorin-susceptible (BSS) clone and the Samorin-resistant (SR) clone were derived from the protozoon isolated in Chisamba. The BSS-clone had MCD values of 7 mg/kg Berenil and 0.5 mg/kg Samorin and it was maintained under drug-free condition. The SR-clone was derived from a sub-population of BSS-clone by administration of repeated sub-curative Samorin dose to infected mice. After six months, it became resistant to Samorin with a new MCD value of 16 mg/kg but it remained susceptible to 7 mg/kg Berenil.

Four groups of twenty ddY mice weighing 20–30 g each were chosen and intraperitoneally (i.p.) inoculated with each of the trypanosome clones. In the Berenil-treatment groups, one group was inoculated with BR-clone and the other with BSS-clone. In the Samorin-treatment groups, one group was inoculated with SR-clone and the other with BSS-clone. The infected mice were monitored daily for parasitemia. At $\geq 5 \times 10^5$ trypanosomes/*ml* of blood (6 to 7 days post-inoculation) the treatments were commenced. Three subgroups of five mice each in each of the Berenil-treatment groups were treated (i.p.) with 3.5, 14.0, or 45.0 mg/kg Berenil. Similarly, three subgroups of five mice each in each of the Samorin-treatment groups were treated with a 0.5, 4.0, or 16.0 mg/kg Samorin. Five mice inoculated only with distilled water in each group, served as control. Berenil and Samorin solutions were prepared by dissolving the required quantities of each compound in sterile distilled water just before use.

Just before treatment (0 hr) and 4, 10, 24 and 48 hr post-treatment, wet blood film preparation from each mouse was examined for parasitemia to determine the efficacy of the drug doses used. At 10 hr post-treatment, ten thin blood smear slides for Ak-counts were also prepared from the peripheral blood of each treated mouse [3]. Aparasitemic mice were monitored for at least 8 weeks for any relapses before being declared as permanently cured. Five of the slides from each mouse were fixed in methyl alcohol for 10 min, dried and hydrolyzed at 60°C in 1 N HCl for 3 min, washed and stained for 45 min with 5% Giemsa solution. Ak-induction rates at 10 hr after the treatment for each mouse were derived by determining the percentage of the counts of Ak-forms in a total count of at least 500 trypanosomes per slide, and the final group average of the Ak-rates (n=5) was thus established. The slides were examined using a light microscope (Nikon UFX II A System). The other five slides from each mouse, were fixed in methyl alcohol for 10 min, air dried and hydrolyzed in 1 N HCl for 3 min as before. After washing, the slides were stained for 8 min in 4,6 diamidino-2-phenylindole (DAPI) staining solution (1 μg DAPI / *ml* distilled water). This staining was followed

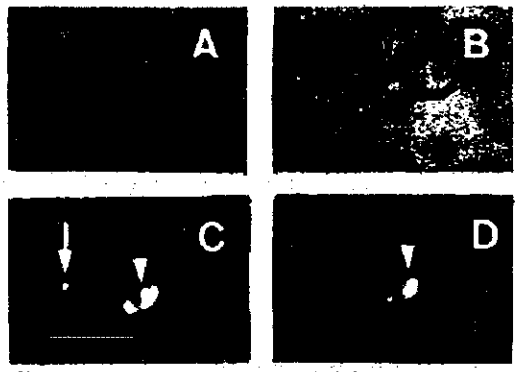

Fig. 1. Intact (A) and AK-forms (B) of *Trypanosoma congolense* following 60 min of modified Giemsa staining. Intact (C) and AK-form (D) of the same species following 20 min DAPI fluorencent staining. Arrow and arrow-head show the kinetoplast and nucleus, respectively. (Bar scale = 11 μm at × 1,000 mag.)

by a two minute-double staining with DAPI solution containing one percent mercaptoethanol before rinsing by dipping in clean water for one to two seconds. The air dried slides were examined directly without cover-slips or oil immersion, using a fluorescent microscope (Nikon, Japan) with a BV 400 nM filter at × 400 magnification. The Ak-rates were again estimated as before, and photographs were taken at a × 1,000 magnification.

The Ak-forms were clearly distinguished from the intact blood stream-forms both in the Giemsa stained (Fig. 1A and 1B) and, in the DAPI stained (Fig. 1C and 1D) slides. It was also possible to accurately predict the dose efficacies in 98% of the treated mice from the Ak-rates. Consistent correlations between Ak-rates and dose efficacies, with respect to trypanosome sensitivities, were observed in both the Berenil- (Tables 1 and 2) and Samorin- (Tables 3 and 4) treatment groups. In general, all mice at 0 h and all the non-treated control mice had Ak-rates of 0.0 to 0.1%. In contrast, in the treatment doses which had no effect on the parasitemia, Ak-rates were 30.0% or less, and in the treatment doses which resulted in temporary clearance of parasitemia accompanied with subsequent relapse, Ak-rates ranged from 30.0 to 50.0%. Whereas, in the treatment doses which resulted in permanently effective cure, Ak-rate values were 50.0% or more.

Table 1. Responses of the BR clone-infected mice to Berenil treatment: a comparison of Giemsa and DAPI staining

Dose[a] (mg/kg)	10 hr AK Values (%)[b] Giemsa	DAPI	Dose efficacy[c]
0(Control)[d]	0.1±0	0	NA
3.5[e]	25.7±2	26.8±2	NE(0/5)
14.0[f]	36.0±2	37.0±2	TE(0/5)
45.0[g]	67.3±2	72.0± .5	PE(5/5)

a) Doses with different designation (d, e, f & g) had significantly different AK-values, P<0.01 (Student *t*-test).
b) Mean±Standard deviation (n=5).
c) NA, not applicable; NE, no effect on clearance of parasitaemia; TE, temporal clearance of parasitaemia resulting in relapse; PE, permanently effective cure with no relapse seen. Numbers in parentheses mean mice per total mice treated.

Table 2. Responses of the BSS strain-infected mice to Berenil treatment: a comparison of Giemsa and DAPI staining

Dose[a] (mg/kg)	10 h AK Value (%)[b] Giemsa	DAPI	Dose efficacy[c]
0 (Control)[d]	0	0	NA
3.5[e]	36.5±1	38.5±2	TE
14.0[e]	63.3±2	64.8±2	PE
45.0[f]	62.5±2	64.9±2	PE

a) Doses with different designation (d, e & f) had significantly different AK values, P<0.01 (Student *t*-test). For explanations of b) & c), please refer to Table 1.

Table 3. Responses of the SR clone-infected mice to Samorin treatment: a comparison of Giemsa and DAPI staining

Dose[a] (mg/kg)	10 h AK Value (%)[b] Giemsa	DAPI	Dose efficacy[c]
0 (Control)[d]	0.1±0	0	NA
0.5[e]	9.5±5	11.5±4	NE(0/5)
4.0[f]	26.0±3	28.5±3	NE(0/5)
16.0[g]	54.4±3	55.1±3	PE(5/5)

a) Doses with different designation (d, e, f, & g) had significantly different AK-values, P<0.01 (Student *t*-test). For explanation of b) & c), please refer to Table 1.

Table 4. Responses of the BSS clone infected-mice to Samorin treatment: a comparison of Giemsa and DAPI staining

Dose[a] (mg/kg)	10 h AK Value (%)[b] Giemsa	DAPI	Dose efficacy[c]
0 (Control)[d]	0	0	NA
0.5[e]	55.2×3	56.6±2	TE/PE(4/5)
4.0[e]	60.3±3	62.2±2	PE(5/5)
16.0[e]	61.0±2	64.5±1	PE(5/5)

a) Doses with different designation (e & f) had significantly different AK-values, P<0.01 (Student *t*-Test). For explanation of b) & c), please refer to Table 1.

Since DAPI was synthesized as a trypanocidal compound [4], it has also been found useful as a DNA stain in various micro-organisms [1, 7, 17, 20]. DAPI has a high affinity and specificity for double stranded DNA regions rich in thymine and adenine [6] to give the DNA-DAPI complex its unique fluorescence staining properties. In this study, we have found DAPI to be useful for rapid

detection of Ak-forms. It facilitated a quick and easy slide processing which substantially reduced the overall time from about 60 min in Giemsa staining to only about 20 min in DAPI staining. Under a fluorescent dark field (Fig. 1C), a trypanosome stained with DAPI showed a more distinct kinetoplast and nucleus than those in Giemsa-stained one even at low magnification (× 400). In the Giemsa-stained slides, the kinetoplast could be clearly detected only at high magnification (× 1,000) using an oil immersion objective lens. This makes DAPI staining more convenient when protozoans are in low parasitemia and a sample of large size has to be examined. Some trypanosomes contain chromophil-like cytoplasmic granules which stains similar to the kinetoplast in Giemsa staining. HCl-hydrolysis helps to remove such granules and free RNA [Ono, personal communication]. The HCl-treatment was also essential for reducing the non-specific back ground effects and for enhancing the fluorescence of trypanosomes in the DAPI fluorescence microscopy.

The use of DAPI staining in the Ak-induction test is also economically feasible for wide-scale application because this stain makes fluorescent microscopic observation possible without use of conventional reagents such as buffers, mono-clonal antibodies, conjugates and others. Above all, only one milligram of DAPI prepares enough staining solution for more than one thousand slides. Moreover, the DAPI solution has economical advantage in that it can be successfully stored for three weeks or more under refrigeration (4°C) and in the dark.

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Trypanosoma congolense: Manifestation of Resistance to Berenil and Samorin in Cloned Trypanosomes Isolated from Zambian Cattle

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Summary

Four *Trypanosoma congolense* clones derived from a Mumbwa field isolate proved to be resistant to Berenil with a minimum curative dose (MCD) value of 40 mg/kg and to Samorin with an MCD of 4 mg/kg for mice. Two other clones, one being resistant to Berenil with an MCD of 45 mg/kg but susceptible to 1 mg/kg Samorin, and the other being resistant to Samorin with MCD of 16 mg/kg but susceptible to 7 mg/kg Berenil, were experimentally rendered resistant to each of the respective drugs they were susceptible to by subcurative treatments in mice. The original trypanosome strains and their derivative clones were then screened for their sensitivity to Berenil or Samorin. Three clones derived from the Mumbwa isolate were resistant to Berenil, with MCD's of 14 to 28 mg/kg, and to Samorin, with MCD's of 4 mg/kg. A single Mumbwa derivative clone was relatively sensitive to both Berenil with an MCD of 7 mg/kg and to Samorin with an MCD of 2 mg/kg. The reciprocal drug induction results confirmed that although trypanosomes can acquire tolerance to both Berenil and Samorin, no cross-resistance between the two was evident.

Zusammenfassung

Vier aus einem im Felde gewonnenen Isolat (Mumbwa) stammende Klone von *Trypanosoma congolense* erwiesen sich als resistent gegenüber Berenil bei einer minimalen kurativen Dosierung (MCD) von 40 mg/kg und gegenüber Samorin mit einem MCD-Wert von 4 mg/kg bei der Maus. Zwei andere Klone, von denen der eine mit einem MCD-Wert von 45 mg/kg gegen Berenil resistant, aber bei 1 mg/kg für Samorin empfänglich war und der andere gegen Samorin resistent (MCD 16 mg/kg) und für Berenil empfänglich (7 mg/kg) war, wurden experimentell jeweils gegen die entsprechenden Arzneimittel resistent gemacht, bei denen sie in der Maus eine Empfänglichkeit bei subkurativer Behandlung zeigten. Die ursprünglichen Trypanosomen-Stämme und die von ihnen abgeleiteten Klone wurden dann auf ihre Empfindlichkeit gegenüber Berenil bzw. Samorin geprüft. Drei aus dem Mumbwa-

Isolat stammende Klone waren gegen Berenil mit MCD-Werten von 14–28 mg/kg und gegen Samorin mit MCD-Werten von 4 mg/kg resistent. Ein einziger Mumbwa-Klon war sowohl gegen Berenil (MCD 7 mg/kg) als auch gegen Samorin (MCD 2 mg/kg) relativ empfindlich. Die Ergebnisse der wechselseitigen Induktion durch die Mittel bestätigten, daß, obwohl Trypanosomen eine Toleranz gegen Berenil und Samorin erwerben können, sich keine Kreuzresistenz zwischen beiden zeigte.

Introduction

The use of trypanocidal drugs to control animal trypanosomiasis in Africa is certainly enormous (6, 17, 22). However, almost all the commonly used trypanocides are gradually losing their efficacy because of the emergence of drug resistance (37, 38, 39). Among the few effective drugs now in use, diminazene aceturate (Berenil), a curative drug, and isometamidium chloride (Samorin), a prophylactic and curative drug, assume particular importance (6).

The occurrence of resistance to one or more trypanocidal compounds in trypanosomes has been a serious concern for a long time (3, 7, 11, 15, 18, 28, 29, 32, 35). In certain cases, ethidium and dimidium compounds were reported to have induced resistance in trypanosomes following a single treatment of cattle infected with *Trypanosoma congolense* and *T. vivax* (36). Although cross-resistance to Berenil, melarsoprol and/or quinapyramine was reported in the 1960's (18, 29, 32), resistance to Berenil or Samorin, which were then still relatively new compounds, was reportedly difficult to establish either experimentally in the laboratory or in the field (2, 12, 13, 14, 17, 31, 32, 34). Cross-resistance between Berenil and Samorin was considered to be an even more difficult occurrence (13, 14, 36). Thus, following recommendations (31, 32), it became customary to use Berenil and Samorin as a sanative pair of drugs for treatment of animal trypanosomiasis. These drugs are being alternatively used on a very wide scale to raise cattle in trypanosomiasis risk areas of Africa even today (4).

However, trypanosomes resistant to Berenil and/or Samorin, have since been suspected to be insidiously developing in the field after more than four decades of extensive use of these drugs (27). Indeed, certain recent field isolates have began to exhibit tolerance to both Berenil and Samorin (1, 9, 26, 27, 40). Interpretation of such double tolerance observed, has remained rather unclear because it may be attributed to the presence of truly double-resistant strains and/or merely to the presence of various mixed strains in a given population which exhibit tolerance to either Berenil or Samorin (25).

It was recently confirmed that trypanosome can truly be resistant to both Berenil and Samorin (25). This indicates a very serious development which may adversely affect the future of trypanosomiasis control by a chemotherapeutic approach using these trypanocides. Such a development will certainly require alternative strategies in the overall management of these drugs to avoid and/or to minimize a possible rampant development of double resistance. Particularly in areas where excessive use of these drugs is common, usually in the absence of any qualified veterinary supervision, as is often witnessed in Africa, such strategies are needed.

As a prerequisite for such an undertaking, it is necessary to first establish the present status of this problem in the field. In our previous drug sensitivity screening study, involving four trypanosome field isolates from Zambian cattle, a certain isolate from Mumbwa exhibited high tolerance to both Berenil and Samorin (9). However, the

results were inconclusive with regard to resolving the actual factors tributing to the apparent double tolerance observed.

The main objectives in this study were twofold:

(1) to determine whether or not trypanosomes truly resistant to both Berenil and Samorin exist in four single trypanosome-derived clonal populations from the original Mumbwa field isolate.

(2) to study the curative effect of Berenil and Samorin using a reciprocal experimental drug-resistance induction approach in mice. Two trypanosome clones of well established resistance to Berenil or Samorin, but reciprocally susceptible to each other, were used. These clonal population strains were derived from the original Chipata and Chisamba field isolates (9).

Material and Methods

Drugs. Diminazene aceturate (Berenil, Hoechst) and isometamidium chloride (Samorin, May and Baker), were prepared by dissolving the required quantities of each drug in sterile distilled water just before use.

Animals. Ila : ddY mice (20–30 g) kept in a fly-proof isolation building were used in both the cloning and drug sensitivity tests. Water and pellet feed were provided ad libitum.

Trypanosomes. All strains of the representative *Trypanosoma congolense* populations used in these experiments had been originally isolated from Zambian cattle (9). Cloning was carried out according to the established hanging drop technique (21). No irradiation of recipient mice was used.

Designation of the trypanosome strains. LusTat 2.0 was a single-trypanosome derived clonal population from the original Chisamba field isolate. It was proved to be sensitive to both Berenil and Samorin (BSS-strain), with minimum curative dose (MCD) values (minimum dose necessary to achieve 100% cure in at least 3 mice) of 7.00 mg/kg Berenil and 0.5 mg/kg Samorin in mice. This strain served as an overall sensitive control.

MubTat 1.0 was the original, uncloned, Mumbwa field isolate. It was proved to be resistant to both Berenil and Samorin (BSR strain), with MCD values of 40.0 mg/kg for Berenil and 4.0 mg/kg for Samorin (9).

MubTat 1.1, 1.2, 1.3 and 1.4 were four single-trypanosome derived clonal populations prepared from the original MubTat 1.0 field population.

ChiTat 1.0 was a single-trypanosome derived clonal population from the original Chipata field isolate (9). This clone was proved to be resistant to Berenil (BR strain) with an MCD value of 45 mg/kg, but it was susceptible to 1 mg/kg Samorin (SS strain) in mice.

ChiTat 1.1 was then derived by experimentally inducing the ChiTat 1.0 strain to become resistant to Samorin under drug pressure, achieved by administering repeated sub-curative treatment doses in mice.

LusTat 1.1 was derived from LusTat 1.0, i.e. another clonal population prepared from the original Chisamba field isolate which was susceptible to 7 mg/kg Berenil and 0.5 mg/kg Samorin (BSS-strain) in mice. Experimental induction of resistance to Samorin under drug pressure in mice (10) resulted in the acquisition of resistance to Samorin (SR-strain) with an MCD value of 16 mg/kg. This strain remained susceptible to 7 mg/kg Berenil (BS strain), it was designated LusTat 1.1.

LusTat 1.2 was then derived by inducing LusTat 1.1 to become resistant to Berenil under drug pressure in mice.

Experimental Procedures

Experiment 1

Fifteen (15) mice were inoculated intraperitoneally (i.p.) with LusTat 2.0 (BSS strain) control. When parasitaemia reached $\geq 5 \times 10^5$/ml of blood as estimated from the peripheral blood counts using wet film preparations (21), four subgroups of 3 mice each were treated (i.p.) with 0.5 to 1.0 mg/kg Samorin or 3.5 to 7.0 mg/kg Berenil. Three mice inoculated only with distilled water, served as zero-treatment controls.

Sensitivity test for Mumbwa clonal populations. Two groups of 18 mice were allotted and inoculated (i.p.) with each of the trypanosome strains; MubTat 1.0, 1.1, 1.2, 1.3, or 1.4. When parasitaemia reached $\geq 5 \times 10^5$/ml of blood, mice in the first group were treated with Berenil, while mice in the second group were treated with Samorin. In the Berenil treatment groups, five subgroups of 3 mice each were treated (i.p.) with 3.5, 7.0, 14.0, 28.0, or 56.0 mg/kg of Berenil. Similarly, five subgroups of 3 mice each in the Samorin treatment groups, were treated with 0.5, 1.0, 2.0, 4.0 or 8.0 mg/kg of Samorin. The MCD values for each strain were thus established. In all the treatment groups, three mice served as zero-treatment controls.

Experiment 2

Reciprocal drug resistance induction test. Two groups of 3 mice each were inoculated (i.p.) with ChiTat 1.0 (BR strain) or LusTat 1.1 (SR strain). Inoculated mice were monitored for the onset of parasitaemia by examining the peripheral blood every other day. At parasitaemia levels of $\geq 5 \times 10^6$/ml of blood, ChiTat 1.0-infected mice were given Samorin treatment at 0.5 mg/kg, while the LusTat 1.1 infected mice were treated (i.p.) with 3.5 mg/kg Berenil. From each mouse, 0.1 ml highly parasitaemic blood (about 5×10^5 parasites) was drawn from the tail vein into phosphate glucose saline, pH 7.5 (1:2 v/v). Pooled parasitaemic blood from each clone was then passaged (i.p.) into another 3 mice at intervals of 30 min, 1, 2 and 6 h post-treatment. This procedure was repeated several times while gradually increasing the initial doses by doubling up to 14.0 mg/kg for Berenil and 2.0 mg/kg for Samorin (with at least five passages at each dose level). Thereafter, all mice with relapsing parasitaemia were repeatedly treated with either 7 mg/kg Berenil or 1 mg/kg Samorin, with only occasional passaging.

Following 5 to 6 months of rapid serial passages after short drug exposure, and the subsequent subcurative treatment doses, the most profilic derivative strains with tolerance for Berenil and Samorin. ChiTat 1.1 (BSR-Strain) and LusTat 1.2 (SBR-Strain) were selected. Both the original strains (ChiTat 1.0 and LusTat 1.1), and their derivative strains (ChiTat 1.1 and LusTat 1.2) were then screened for Berenil and Samorin sensitivity to determine the resultant MCD values. Except for the two Samorin treatment groups inoculated with LusTat 1.1 and LusTat 1.2 strains (Table 6), to which 21 mice had been alloted and given an extra dose of 16.0 mg/kg Samorin, the treatment protocol used in all the other Berenil and Samorin treatment groups was identical to the one outlined for MubTat strains in Experiment 1.

Determination of drug efficacy in mice. At 10 h post-treatment, five thin blood smear slides were prepared from each mouse fixed in alcohol and stained according to Giemsa before examination under a light microscope. The dose efficacy values were determined by establishing the akinetoplastic (Ak) blood stream form (trypanosomes with no detectable kinetoplast) induction rates as a percentage of the total trypanosome counts (10). At least 500 trypanosomes/slide were counted under X1000 magnification and the Ak values were expressed as the average of the percentages ($n = 5$). In general, 10 h Ak values of $< 30\%$ represented non-effective dose response, Ak values between 30 and 50% respresented temporary cure resulting in relapsing parasitaemia, while Ak values of $> 50\%$ represented curative doses. All the treated mice were checked to confirm parasitaemia elimination by examining the peripheral blood using wet film preparations, starting at 24 and 48 h post-

treatment and on every other day for at least 60 days. Mice which remained aparasitaemic after the 60-day observation period, were declared permanently cured.

Results

All zero-treatment control mice, had Ak values ranging from 0.0 to 0.5% in all the treatment groups.

Experiment 1.

Judging from the results obtained using the Ak induction method or the conventional mouse model approach, it was possibe to clearly establish the sensitivity profiles between susceptible and resistant trypanosomes following treatment with Samorin or Berenil. LusTat 2.0 strain had MCD values of 7.0 mg/kg for Berenil and 0.5 mg/kg for Samorin (Table 1). These doses corresponded to 10 h Ak values of 50% or more. Only

Table 1. Responses of mice infected with LusTat 2.0 (BSS strain) to curative doses of Samorin (S) and Berenil (B) (Experiment 1)

Group (No.)	Mice (No.)	Dose (mg/kg)	Dose efficacy	Ak values (% Avg. ± SD)	Avg. days to relapse (No.)	Mortality (%)
1	3	Control	NA	00.00	NA	100**
2	3	0.5 S	PE	59.67 ± 3	NA	0
3	3	1.0 S	PE	63.26 ± 4	NA	0
4	3	3.5 B	TE	37.30 ± 5	13.1 ± 2	100*
5	3	7.0 B	PE	65.03 ± 2	NA	0

Na, not applicable; TE, temporary effect; PE, permanent cure.
** Mice survived for an average of 25 days after onset of parasitaemia.
* Mice survived for an average of 45 days after following relapsing parasitaemia.

a 3.5 mg/kg Berenil dose resulted in a relapsing parasitaemia in this group, 2 weeks post-treatment. This relapsing dose corresponded to a 10 h Ak value of 37%.

Results for MubTat 1.0 and its derivative clones have been summarized in Table 2. Although no clone exhibited the same Berenil MCD value as the MubTat 1.0 strain, variation in MCD values obtained following Berenil and Samorin treatments was evident. MubTat 1.0 had MCD values of 56 mg/kg for Berenil and 4.0 mg/kg for Samorin, which corresponded to Ak values of more than 50% for each of the two drugs. The MubTat 1.1 clone was tolerant for both Berenil and Samorin, with MCD values of 14.0 mg/kg for Berenil and 4.0 mg/kg for Samorin. This corresponded to 10 h Ak values of 59% and 69% for Berenil and Samorin, respectively. The MubTat 1.2 clone was also tolerant for both Berenil and Samorin. It had a Berenil MCD value of 14.0 mg/kg and a Samorin MCD value of 4.0 mg/kg, which corresponded to 10 h Ak values of 57% for Berenil and 56% for Samorin. The MubTat 1.3 clone was found to be relatively more sensitive to Berenil, with an MCD value of 7.0 mg/kg, and to Samorin, with an MCD value of 2 mg/kg, which corresponded to 10 h Ak values of

Table 2. Summary of the response of MubTat 1.0 derivative clones to various Berenil or Samorin treatment doses (Experiment 1)

Clone derivative	No. of mice/ sub-group	MCD values (mg/kg)		Ak values (% avg + SD)	
		Berenil	Samorin	Berenil	Samorin
MubTat 1.0	3 (18 X 2)	56.00	4.0	57.15 ± 5	60.44 ± 4
MubTat 1.1	3 (18 X 2)	14.00	4.0	59.05 ± 5	69.44 ± 4
MubTat 1.2	3 (18 X 2)	14.00	4.0	56.55 ± 5	55.87 ± 2
MubTat 1.3	3 (18 X 2)	7.00	2.0	54.60 ± 5	56.50 ± 3
MubTat 1.4	3 (18 X 2)	28.00	4.0	55.30 ± 5	55.67 ± 5

NB: Berenil was administered at 0, 3.5, 7.0, 14.0, 28 or 56.0 mg/kg/group, while Samorin was administered at 0.0, 0.5, 1.0, 2.0 4.0 or 8.0 mg/kg/group.
MCD values represent minimal curative dose reached to achieve permanent cure in all the 3 treated mice/sub-group.
Figures in parentheses represent total number of mice used for each strain.

55% for Berenil and 57% for Samorin. In contrast, the MubTat 1.4 clone was more tolerant for both drugs; it had MCD values of 28.0 mg/kg Berenil and 4.0 mg/kg Samorin, which corresponded to 10 h Ak values of 55% for Berenil and 56% for Samorin.

Experiment 2

Results from the reciprocal drug-induction experiments showed that the Berenil-resistant clone (ChiTat 1.0) had acquired more tolerance for Samorin (ChiTat 1.1), with a new MCD value of 4.0 mg/kg from a dose of 1 mg/kg, which corresponded to the 10 h Ak value of 66% (Table 3). Nevertheless, it retained its resistance to Berenil, with an MCD value of 56.0 mg/kg which corresponded to 10 h Ak value of 67% (Table 4). Similar results were also observed in the Samorin-resistant clone (LusTat 1.1) responses. It acquired more tolerance to Berenil (LusTat 1.2), with a new MCD

Table 3. Responses to Samorin doses of ChiTat 1.0 (BR-strain) and ChiTat 1.1 (BSR-strain) before and after reciprocal Samorin resistance induction, respectively (Experiment 2)

Group	Dose (mg/kg)	Mice/ group	Before (ChiTat 1.0)		After (ChiTat 1.1)	
			Ak value (% avg.)	Efficacy	Ak value (% avg.)	Efficacy
1	Control	3 X 2	00.00	NA	00.00	NA
2	0.5	3 X 2	36.10 ± 2	TE	31.91 ± 3	TE
3	1.0	3 X 2	51.10 ± 2	PE	41.22 ± 5	TE
4	2.0	3 X 2	62.20 ± 4	PE	45.85 ± 3	TE
5	4.0	3 X 2	70.2 ± 5	PE	65.91 ± 3	PE
6	8.0	3 X 2	NPS	PE	69.31 ± 3	PE

NA, not applicable; TE, temporary effect; PE, permanent effect; NPS, no parasite seen.

Table 4. Responses to Berenil doses of ChiTat 1.0 (BR-strain) and ChiTat 1.1 (BSR-strain) before and after reciprocal Samorin resistance induction, respectively (Experiment 2)

Group	Dose (mg/kg)	Mice/ group	Before (ChiTat 1.0)		After (ChiTat 1.1)	
			Ak value (% avg.)	Efficacy	Ak value (% avg.)	Efficacy
1	Control	3 X 2	0.50 ± 0.1	NA	00.00	NA
2	3.5	3 X 2	28.00 ± 1	NE	15.43 ± 2	NE
3	7.0	3 X 2	34.50 ± 2	TE	36.71 ± 4	TE
4	14.0	3 X 2	37.90 ± 1	TE	42.35 ± 3	TE
5	28.0	3 X 2	40.30 ± 2	TE	53.55 ± 2	TE/PE (1/3)*
6	56.0	3 X 2	65.00 ± 4	PE	67.40 ± 5	PE

NA, not applicable; TNE, no effect; TE, temporary effect; PE, permanent effect.
* No. of mice permanently cured / No. of mice treated.

value of 28.0 mg/kg from a dose of 7 mg/kg, which corresponded to an Ak value of 55% (Table 5). It also retained its resistance to Samorin with an MCD value of 16.0 mg/kg, which corresponded to a 10 h Ak value of 63% (Table 6).

It was also generally observed that survival rates in the zero-treatment control mice varied depending on the trypanosome strain. For example, mice infected with LusTat 2.0, a less virulent strain, survived longer (avg. of 25 ± 8 days) even in the absence of any treatment. On the other hand, all resistant strains were more virulent. They killed untreated mice within 7 to 14 days after onset of parasitaemia. Mortality in mice following relapsing parasitaemias to Berenil or Samorin treatments, also varied depending on the dose used: The higher the dose, the longer the mice survived (avg. of 30 ± 14 days).

Table 5. Responses to Berenil doses of LusTat 1.1 (SR-strain) and LusTat 1.2 (SBR-strain) before and after reciprocal Berenil resistance induction, respectively (Experiment 2)

Group	Dose (mg/kg)	Mice/ group	Before (LusTat 1.1)		After (LusTat 1.2)	
			Ak value (% avg.)	Efficacy	Ak value (% avg.)	Efficacy
1	Control	3 X 2	00.00	NA	00.00	NA
2	3.5	3 X 2	38.10 ± 3	TE	23.61 ± 4	TE
3	7.0	3 X 2	52.20 ± 2	PE	31.03 ± 2	TE
4	14.0	3 X 2	62.40 ± 3	PE	48.10 ± 3	TE
5	28.0	3 X 2	80.00 ± 5	PE	55.00 ± 4	PE
6	56.00	3 X 2	NPS	PE	NPS	PE

NA, not applicable; TE, temporary effect; PE, permanent effect; NPS, no parasite seen.

Table 6. Responses to Samorin doses of LusTat 1.1 (SR-strain) and LusTat 1.2 (SBR-strain) before and after reciprocal Berenil resistance induction, respectively (Experiment 2)

Group	Dose (mg/kg)	Mice/ group	Before (LusTat 1.1)		After (LusTat 1.2)	
			Ak value (% avg.)	Efficacy	Ak value (% avg.)	Efficacy
1	Control	3 X 2	00.00	NA	00.00	NA
2	0.5	3 X 2	10.20±0.1	NE	32.10±4	TE
3	1.0	3 X 2	19.80±2	NE	38.42±2	TE
4	2.0	3 X 2	31.00±3	NE	45.33±5	TE
5	4.0	3 X 2	43.00±2	TE	49.32±2	TE
6	8.0	3 X 2	48.70±4	TE	52.41±3	TE/PE (2/3)*
7	16.0	3 X 2	53.50±2	PE	62.50±3	PE

NA, not applicable; TNE, no effect; TE, temporary effect; PE, permanent cure.
* No. of mice permanently cured / No. of mice treated.

Discussion

Trypanosome resistance to drugs is closely associated with areas where trypanocidal drugs are widely used (12, 39). Development of drug resistance in trypanosomes has not yet been well elucidated, but it has been suggested that possible mechanisms could be similar to those reported for the induction of antibiotic resistance in bacteria (3). However, the number of trypanocidal drugs to which a single trypanosome can become directly resistant to is also not very well established.

Prolonged and excessive use of Berenil and Samorin on a routine herd basis is a common feature in African livestock husbandry practices. The use of these trypanocidal drugs is particularly intensive among the commercially managed herds, in comparison with management in the traditional sector. Because most of these animals are located in high risk areas, they are almost always infected with trypanosomiasis. Trypanosomes are therefore often in a state of continuous exposure to trypanocidal drugs which in turn constitute an ideal pre-condition for the development of drug resistant strains (3, 38, 39). Once a few strains have developed resistance to a given drug, subsequent clonal expansion of such trypanosomes may give rise to a phenomenon of mixed strains exhibiting varying drug sensitivity profiles within a given field population (30). Inadequate veterinary supervision of farmers and the inappropriate methods currently in use for monitoring such trypanosomes under field conditions may also contribute to the rampant development of drug resistant strains observed in recent field isolates (1, 9, 20, 26, 27, 39).

Although Berenil is known to act through precipitation of nucleotide phosphatases and other nucleotide co-enzymes (19), the mode of action for both Berenil and Samorin is mainly associated with functional interference in the trypanosome DNA synthesis (3, 5, 6, 23, 24). In spite of the apparent similarities in their modes of action, cross-resistance between the two is evidently non-existent (2, 13, 14, 16, 32, 34). Nevertheless, a certain multiple resistant *T. brucei* stock which was found to be resistant to all the commonly used trypanocides except Suramin, was also reportedly tolerant to five experimental trypanocidal compounds without any pre-exposure (40).

Results obtained in the present study have confirmed that the observation of trypanosomes with double tolerance to Berenil and Samorin as earlier seen in the Mumbwa field isolate from Zambian cattle was, in part, attributable to both the presence of multiple resistant strains and to the presence of mixed trypanosome strains with different sensitivity to Berenil or Samorin. The present findings are in agreement with a recent report on the existence of trypanosome clones showing multiple resistance to both Berenil and Samorin (25).

The results of experiment 2 clearly show an independent acquisition of resistance to Berenil and Samorin rather than a cross-resistance between the two. Our observations seem to suggest that virulence in trypanosomes may be enhanced following acquisition of drug resistance. The present results also support the idea that any prolonged indiscriminate use of both Berenil and Samorin in a herd can eventually lead to a gradual acquisition of resistance to these drugs, as manifested in the Mumbwa isolate. This problem of multiple resistance is likely to be associated with commercially managed herds, which are generally maintained under prolonged intensive use of both Berenil and Samorin. In contrast, in the traditionally managed herds which exhibit relatively greater trypanotolerance and are rarely maintained under trypanocidal protection, may be less likely affected by this development. Indeed, this problem could be exacerbated by a high trypanosomiasis challenge.

Development of resistance to both Berenil and/or Samorin is apparently a slow process even under experimental drug pressure and, cross-resistance between these two drugs has not yet been proved. Therefore, there is hope for their continued use as a curative pair of drugs. Nevertheless, such regimens should be strictly supervised and supported by a more regular monitoring service to examine drug efficacy, which in turn will facilitate a more economical and effective use of these highly priced trypanocides.

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Pasture Infestation with Infective Larvae of *Haemonchus Contortus* on a Sheep Farm in Zambia.

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The population dynamics of the free living stage of gastro-intestinal nematodes have been investigated by several invest-igators mainly in temperate regions (1, 2, 3, 4, 5) and it is suggested that various factors such as temperature, moisture, soil condition, grass etc. can affect the development and longevity of a population of infective larvae on pasture.

Zambia has a rainy warm season from November to March and a dry season from April to October. During the mid and late rainy and early dry season in 1987 and 1988, grass was collected from paddocks of a sheep farm in Lusaka, Zambia where infection with *Haemonchus contortus* had been known. These paddocks were wire-fenced into 9 paddocks each of 200 x 200 m^2 and the flocks, consisting of 60 ewes. were grazed and rotated every few weeks from paddock to paddock. A total of 1 kg of grass was collected from each of 6 paddocks namely A, B, C, D, E and F, collection being made over a zig-zag traverse of each paddock. Date of sampling and period of grazing are shown in the figure. The infective larvae were recovered from the grass samples following the methods of Smeal and Hendy (6). The rainfall data was obtained from Lusaka Meteorology Centre.

All infective larvae recovered were *Haemonchus spp.*. The date of sampling, number of larvae recovered from grass which was collected from each paddock and rainfall data in the period are shown in the figure.

During the 1986/1987 rainy season, although there was stable rainfall in November and early December, rainfall was very scarce during the middle and late rainy season except for a two week period in late January. Outbreaks of acute haemonchosis and several deaths were observed in the period from November to January. High infestation of pasture in these paddocks, with 721 larvae/500 gm. of wet grass sample found in paddock B after 4 weeks grazing. Relatively few larvae were recovered from paddocks D and F where ewes had been removed 2 and 4 weeks respectively before the grass sampling although a considerable number of larvae were found in paddock A after 2 weeks grazing and paddock C and E after 1 and 3 weeks rest respectively.

In 1988, rainfall was relatively stable. Anthelmintic treatment of the ewes by drenching with benzimidazoles was conducted every 3 weeks during the rainy season and larvae on the pasture were fewer than in 1987. At the initial sampling, conducted at the end of the rainy season a few larvae recovered from paddocks A and B after 1 and 2 weeks grazing respectively while there was no rainfall. A significantly high number of larvae were recovered from paddock C after 4 weeks grazing and paddocks D and E after 2 and 4 weeks rest respectively. At the second sampling in the early dry season, fewer or no larvae were recovered from grazing paddocks.

In tropical countries, although temperature is rarely a limiting factor, moisture is much lower in dry season than the minimum monthly rainfall for development of haemonchosis (7). Donald(8) and Walter and Donald(9) suggested that the pre-infective stages of *H. contortus* are more susceptible to desiccation and short-term fluctuations in weather conditions than *Trichostrongylus colubriformis* whereas the larvae which have developed to the infective stage and reached herbage may continue to be recovered from herbage for many weeks with only a gradual decline in numbers. Lyaku *et al.*(10) reported the longevity of infective strongylid larvae in several kinds of African tropical soils and they indicated that infective larvae of Haemonchus were viable for less than 3 weeks in Petri dishes under laboratory conditions.

In our study, a considerable number of infective larvae of *Haemonchus contortus* were recovered from the paddocks rested for 4 weeks and a few larvae still remained in the paddock rested for 8 weeks under field conditions in the rainy season. The high level of contamination with infective larvae of paddock B in 1987 is attributable to the 2 weeks of high rainfall during the four weeks grazing period prior to sampling. There was stable rainfall from 8 to 2 weeks prior to sampling in 1988 and considerable numbers of larvae were recovered from the paddocks after 4 and 8 weeks rest. It is suggested that relatively short term rainfall caused the rapid increase of larvae and stable rain fall prolonged the survival of those larvae up to more than two months. As observed in paddock A, B and C in 1988, continuous aerial grazing under dry conditions did not cause the further accumulation of larvae and the number of larvae decreased to or was sustained at a minimal level of contamination.

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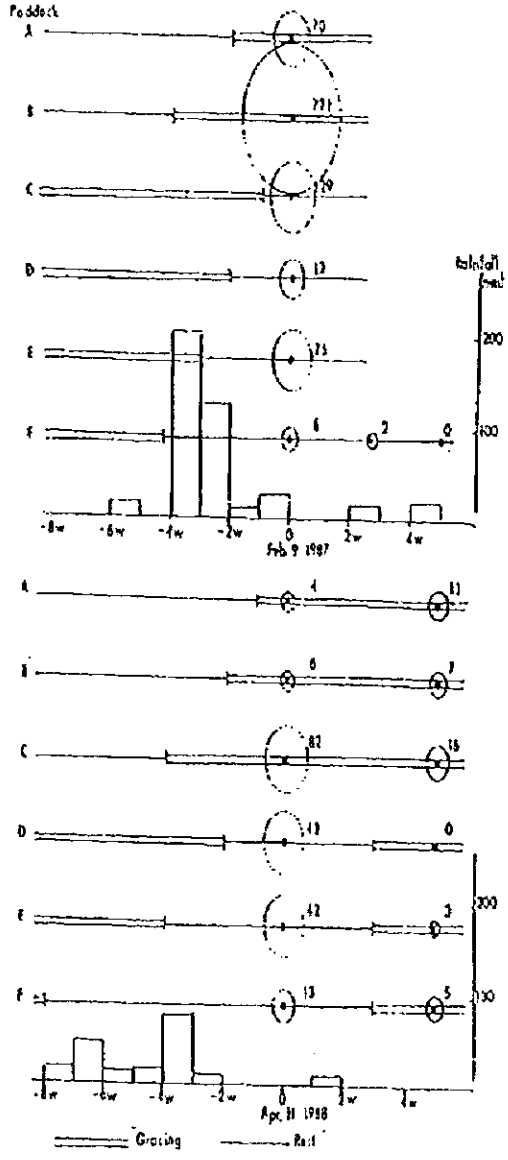
Figure: Number of infective larvae of *H. contortus* recovered from the 500 grams of wet grass sample, collected from the paddock of a sheep farm in Lusaka, Zambia. Dimension of the circle corresponds to the number of infective larvae.



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THEILERIOSIS IN ZAMBIA: ETIOLOGY, EPIDEMIOLOGY AND CONTROL MEASURES.

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ABSTRACT

In Zambia, theileriosis manifests itself in the form of Corridor disease (CD), caused by *Theileria parva lawrencei*, and East Coast fever (ECF), caused by *T. parva parva*. Of the approximately 3 million cattle in Zambia, 1.4 million are at risk to theileriosis. ECF is found in the Northern and Eastern provinces of the country, while CD appears in Southern, Central, Lusaka and Copperbelt provinces. Theileriosis is a major constraint to the development of the livestock industry in Zambia, with losses of about 10,000 cattle per annum. The disease is spreading at a very fast rate, over-flowing its original borders. The epidemiology is complicated by, among other factors, the wide distribution of the tick vector, *Rhipicephalus appendiculatus*, which is found all over the country. The current strategy of relying on tick control and therapeutic drugs as a way of controlling the disease is becoming increasingly difficult for Zambia. This is because both curative drugs and acaricides are very costly. Immunization against theileriosis using the infection and treatment method as a way of controlling the disease is becoming increasingly accepted, provided local *Theileria* stocks are used. This paper reviews the incidence of theileriosis in the last 2 years, 1991 and 1992. It also gives a historical perspective of the disease, epidemiology and control measures presently in use.

Key words: Theileriosis, East Coast fever, Corridor disease, *Theileria parva*, Zambia

INTRODUCTION

Zambia has a human population of about 8.5 million (estimated 1989) and has an area of 751,000 square kilometers. The annual human population growth rate is estimated at 3.5% with a population density of 9.4 per square kilometer. It has a

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cattle population of approximately 3 million, 257,000 of which are dairy cows. It has 80,000 sheep, 420,000 goats, 221,000 pigs and about 14 million chickens [6].

Theilerioses are protozoan infections of wild and domestic *Bovidae* which occur throughout much of the world. Organisms that cause these diseases are protozoan parasites belonging to the genus *Theileria* (Theiler, 1904). Two species of this genus, *T. annulata* and *T. parva* cause severe clinical disease in cattle that impedes dairy and beef farming and its improvement in countries in Africa, Asia and the Middle East. The most economically important species in Africa is *T. parva*. This parasite is transmitted by *Rhipicephalus appendiculatus* (Neumann, 1901) ticks and causes an often fatal, lymphoproliferative disease of cattle in Eastern, Central and Southern Africa. The case fatality rate in fully susceptible animals approaches 100% if not treated. Of the 63 million cattle raised in this region, over 24 million are at risk from *T. parva* infections [12].

Three *Theileria parva* subspecies, namely, *Theileria parva parva*, *Theileria parva lawrencei* and *Theileria parva bovis* are purported to exist. Although serologically and morphologically identical, they can be distinguished based on certain biological and epidemiological characteristics of the parasites in cattle [31]. Most cattle infected with *T. parva parva* die during a phase of high parasitemia, thus allowing ticks to pick up the parasite and transmit it to other cattle [11]. Therefore, *T. parva parva* can be maintained by a cattle population, but *T. parva lawrencei* might not be. This parasite behaviour necessitates the study of *T. parva lawrencei* in buffalo. *T. parva parva* and *T. parva bovis* are primarily transmitted between cattle, however, the disease caused by *T. parva parva* is generally more severe with a greater abundance of parasites and a higher mortality rate than is observed in *T. parva bovis* infections. *T. parva lawrencei* is a buffalo-derived parasite and is similar in virulence to *T. parva parva*, but generally produces fewer intraerythrocytic piroplasms intralymphocytic schizonts during the course of disease in cattle than in *T. parva parva* infections.

There has been a number of problems encountered in the field when immunizing against *T. parva lawrencei* by infection and treatment and culture methods. Young [32], and Radley et al. [24, 25] found that *T. parva lawrencei* immunized cattle were generally resistant to challenge with *T. parva parva*, but *T. parva parva* immunized cattle died on challenge with *T. parva lawrencei* from buffalo. Another example worth mentioning is that cattle challenged with a lethal dose of *T. parva lawrencei* die before the piroplasm stage appears in the blood [32].

Historical background of theileriosis in Zambia

The actual origin of theileriosis in Zambia is not known. It is highly assumed, like in the neighbouring countries of Malawi, Mozambique and Zimbabwe, that classical East Coast fever (ECF) in the Eastern and Northern provinces originated from East Africa (from the then Tanganyika or German East Africa). Several hypotheses exist

on the origin Corridor disease (CD) in the Southern province:

—spread from Eastern / Northern ECF infected areas,

—spread from buffalo, and

—spread from Zimbabwe (the so-called January Disease caused by *T. parva bovis*.).

However, the first recorded case of theileriosis in Zambia was in the Nakonde area of Northern province in 1922. According to the annual reports of the Veterianary Department of Zambia, no cases of the disease were diagnosed within the country from 1928 to 1945. In 1946 theleriosis was diagnosed in Mbala district in Northern province, and in 1947 in Chipata district in Eastern province. Since 1947 theileriosis has spread within the Northern and Eastern provinces, throught much of which it is now established enzootically.

In 1977 / 78, a malignant form of theileriosis was detected in the Hufwa area of Monze district, in Southern province [5]. Based on the criteria set by Neitz [19], this disease was diagnosed as "CD". According to Neitz [19], this form of theileriosis was distinct from classical ECF because: (1) ticks were not infected after feeding on infected cattle, due to the absence of intraerythrocytic piroplasms in the blood of infected cattle: (2) the mortality of cattle in the buffalo inhabited "corridor" area caused when cattle were moved outside the buffalo zone: (3) only a few macroschizonts of small dimensions were found in the tissue smears of infected cattle: (4) splenectomy caused a recrudescence of piroplasm parasitemia: and (5) a carrier state occurred in buffalo in contrast to *T. parva parva* infection in cattle. Neitz [19] considered that these characteristics of the buffalo parasite were enough to toxonomically separate it from *T. parva* so he created a new species, *T. lawrencei* which is now known as *T. parva lawrencei*. It is fairly certain that prior to 1977 Southern province was free of the disease. The disease has since then become endemic in this region, which is an important cattle-raising area containing about 1.2 million head, approximately 45% of the national herd.

Epidemiology of theileriosis in Zambia

Five *Theileria* species and subspecies are known to exist in Zambia. These are *T. parva parva*, *T. parva lawrencei*, *T. mutans*, *T. velifera* and *T. taurotragi*. The most economically important of these are the *T. parva* transmitted mainly by the tick ectoparasite, *R. appendiculatus*. Infection of susceptible cattle with *T. parva parva* results in the virulent form of the disease known as ECF. *T. parva lawrencei* on the other hand, causes mild infections in the African buffalo (*Syncerus caffer*) but causes lethal infections known as CD when transmitted to cattle by ticks which have previously fed on infected buffalo or infected cattle. CD appears in Southern, Central and Lusaka provinces (Fig. 1). Recently, a number of cases have been recorded in the Copperbelt province. ECF is found in the Northern and Eastern provinces of the

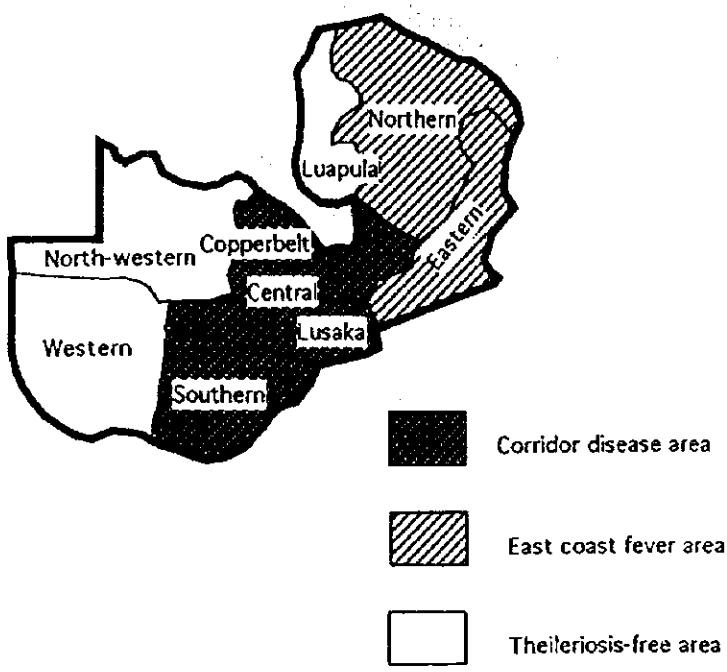

Fig. 1. The distribution of *Theileria parva* in Zambia.

country (Fig. 1). The economic significance of the other three *Theileria* species is not known. Nevertheless, they appear to cause only comparatively mild disease conditions such as transient fever and anemia.

—Factors affecting geographical distribution of theileriosis in Zambia.

Theileriosis persistence in an area is dependent upon the presence, in sufficient numbers of the host, the agent and the vector tick all at the same time [9]. In Zambia, three cattle sub-populations exist: one in the Western and North-western provinces, another in the Sourthern, Central, Lusaka and Copperbelt provinces and the third in the Eastern and Northern provinces. The vector *R. appendiculatus* is found throughout the country even in areas where theileriosis has not yet been reported (Fig. 2). The pattern of seasonal occurrence of *R. appendiculatus* is determined by climate [10, 26, 29]. The seasonal cycle is determined by the adults, which are only active under warm, wet conditions when photophase (day length) exceeds approximately 11 hours [21]. In Zambia, *R. appendiculatus* is, however, most abundant in the highest areas (1000–2000 metres above sea level) with moderate to high rainfall (600mm or more), moderate temperatures (18–30°C), soils which hold sufficient moisture during the dry season and sufficient vegetation cover [27].

In many cattle breeding areas in which vector and host distribution overlap, livestock have either been infected or are at risk of being infected. Within these

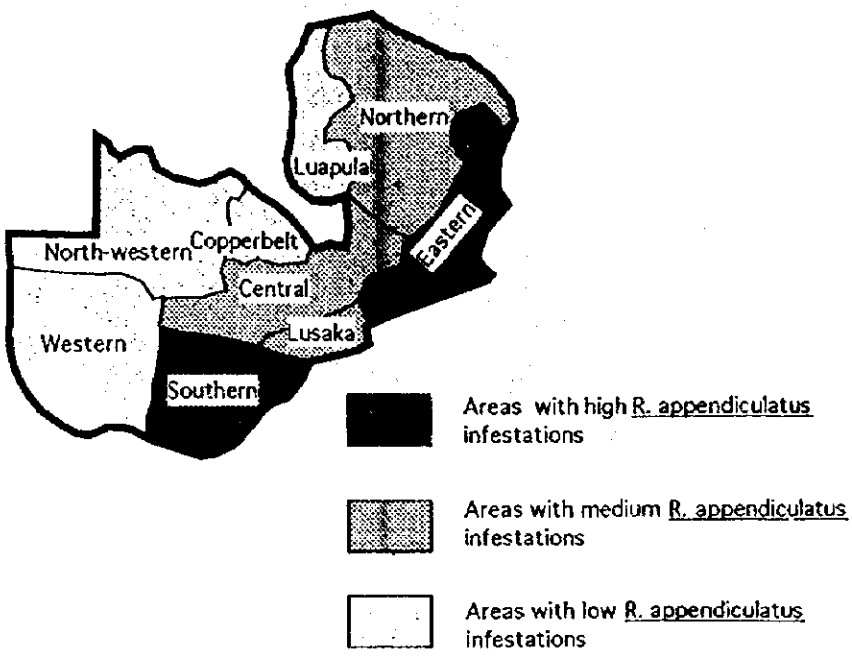

Fig. 2. The distribution of *Phipicephalus appendiculatus*, the vector of *Theileria parva* in Zambia.

areas, cattle populations may be divided into two groups:

(i) cattle kept on enclosed grazing under excellent standards of tick control i.e. commercial and state farms, on which the opportunity for introduction and subsequent spread of theileriosis is minimal as long as the management practices do not deteriorate.

(ii) cattle maintained under poor to moderate vector control standards or under no vector control. Grazing and watering points are communal (traditional sector) or more or less enclosed (settlement schemes). Unlike in other countries where group (i) i.e. commercial sector is most affected, in Zambia, the disease tends to be more active in the latter group.

—Disease patterns and occurrence rates

Depending upon the suitability of local environmental conditions for the vector, traditionally managed herds can be divided into 3 populations:

(i) Populations in areas unsuitable for the vector; here, vector numbers are too low to allow the disease to become established. These are the low-lying valleys of both Southern and Eastern provinces.

(ii) Populations in areas marginal for the vector; here the disease occurs in the form of periodic epizootics of varying size depending upon the climate and other

factors. These are in the areas of intermediate altitude between low-lying and highland areas.

(iii) Populations in areas suitable for the vector; here the abundance of ticks is sufficient to allow maintenance of the disease, the major determining factor being the standard of vector control.

Within an infected area, the pattern of theileriosis occurrence may take the form of a) epizootic b) enzootic occurences and c) enzootic stability.

a) Epizootic occurrence

This is observed in newly-infected heads at the periphery of an infected area, especially in herds in areas marginal for the vector and in herds within enzootic areas in which previously high-level vector control standards have been relaxed allowing the infection to penetrate. Average mortality rates are reportedly in the order of 20% but could be as high as 100% in exceptional cases as recorded in Choma district, Southern province [7].

b) Enzootic occurrence

Under certain conditions, such as moderate standards of vector control, reasonable tick infestation rates, favourable climate, theileriosis occurrence rates decline slowly as more and more animals that survive acquire some resistance to infection, eventually stabilizing in the third to fourth year of infection. Mortality rates may be in the order of 5%–10% with about 16% latent infection [7].

c) Enzootic stability

This may eventually be reached within 4–5 years after first infection (Epidemiology Unit and F.A.O., 1986). Mortalities are mainly in calves and may vary from 0–1.4% [7]. Epizootic may occur in an enzootically stable area if susceptible cattle are brought in. Latent infection may be closer to 100% animals over 12 months old and up to 97% among calves [7].

CURRENT THEILERIOSIS SITUATION IN ZAMBIA

A total of 20,022 theileriosis cases was recorded by the Veterinary Department in 1991 (Tables 2 and 3). Out of which 2,596 were ECF cases with Northern province recording 468 cases, while Eastern province recorded 2,128 ECF cases (Table 4). In 1991 the highest number of ECF cases in both the Northern and the Eastern provinces occurred between January and March (Fig. 3 and Table 3). Rainfall could be an influencing factor as adult *R. appendiculatus* activity is highest during this period. A small peak occurring between April and June could be due to nymphal activity confirming the assertions made previously [1, 20, 22], on the role of *R. appendiculatus* nymphs in the epidemiology of theileriosis. This peak is, however, highly dependant on whether the prevailing climatic conditions, temperature and moisture are favourable for tick development or not. There is a steady decline in the number of ECF cases recorded from June to October. With the onset of the rains in

Table 1. *THEILERIA* SPECIES IN ZAMBIA

Species	Disease caused	Vector
T. parva	East Coast fever	*R. appendiculatus*
T. parva lawrencei	Corridor disease	*R. zambeziensis*
		Rhipicephalus spp.
T. mutans	benign theileriosis	*Amblyomma* spp.
		Rhipicephalus spp.
T. velifera	benign theileriosis	*Amblyomma* spp.
T. taurotragi	benign theileriosis	*R. appendiculatus*
		Rhipicephalus spp.

Table 2. TOTAL NUMBER OF MORTALITIES AND CASES DUE TO CORRIDOR DISEASE IN 1991 & '92 BY MONTH

MONTH	1991		1992	
	CASES	MORTALITIES(%)	CASES	MORTALITIES(%)
JAN.	3,591	1,657(46.1)	2,487	2,008(83.7)
FEB.	1,846	1,072(58.1)	1,482	1,036(69.9)
MAR.	1,041	293(28.0)	1,053	643(61.0)
APR.	1,832	524(28.6)	1,518	952(62.7)
MAY	2,750	1,488(54.1)	2,269	820(36.1)
JUN.	2,961	2,295(77.5)	2,821	518(18.4)
JUL.	990	450(45.5)	1,280	307(24.0)
AUG.	1,046	451(43.1)	559	306(54.7)
SEPT.	391	106(27.1)	221	99(44.8)
OCT.	221	79(35.7)	184	60(32.6)
NOV.	221	60(27.1)	178	22(12.4)
DEC.	536	242(45.1)	542	52(9.6)
TOTAL	17,426	8,717	14,594	6,903

Table 3. TOTAL NUMBER OF MORTALITIES AND CASES DUE TO EAST COAST FEVER IN 1991 & '92 BY MONTH

MONTH	1991		1992	
	CASES	MORTALITIES(%)	CASES	MORTALITIES(%)
JAN.	329	39(11.9)	160	63(39.4)
FEB.	380	66(17.4)	347	48(13.8)
MAR.	468	92(19.7)	272	84(30.9)
APR.	200	25(12.5)	149	48(32.2)
MAY	285	76(26.7)	396	205(51.8)
JUN.	226	26(11.5)	194	104(53.6)
JUL.	191	30(15.7)	114	39(34.2)
AUG.	167	47(28.1)	95	23(24.2)
SEPT.	125	56(44.8)	86	27(31.4)
OCT.	8	0	23	4(17.4)
NOV.	2	0	50	13(26.0)
DEC.	215	58(27.0)	24	3(12.5)
TOTAL	2,596	515	1,910	661

Table 4 TOTAL NUMBER OF MORTALITIES AND CASES DUE TO EAST COAST FEVER IN 1991 & '92 BY PROVINCE

PROVINCE	1991		1992	
	CASES	MORTALITIES(%)	CASES	MORTALITIES(%)
EASTERN	2,128	414(19.5)	1,329	436(32.8)
NORTHERN	468	101(21.6)	581	225(38.7)
TOTAL	2,596	515	1,910	661

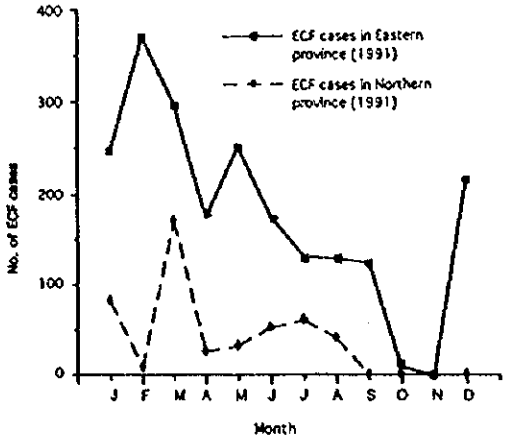

Fig. 3. The number of East Coast fever (ECF) cases in Northern and Eastern provinces as recorded by the Veterinary Department, Lusaka in 1991 by month.

November adult tick activity resumes and the number of ECF cases increases again. The picture was much the same in 1992 in both provinces (Fig. 4).

Of 17,426 CD cases recorded in 1991, 15,409 was in the Southern province, followed by 1,739 in Central province, 152 in the Copperbelt province, and 126 in Lusaka province (Table 2 and 5).

In both 1991 and 1992, the highest number of CD cases in Southern province were recorded during the month of January, (Table 2 and Fig. 5). A small peak occurring between March and July due to high nymphal activity was recorded (Fi. 5). There were, however, CD cases, in low numbers, recorded throughout the year. The epidemiology of CD in Central province in both 1991 and 1992 was rather different from the usual pattern with more cases being recorded between March and August in 1992 (Fig. 6.). A small peak was recorded in the month of November in 1991 and in December in 1992. The Copperbelt province had 152 and 364 recorded CD cases in 1991 and 1992 respectively Tables 4 and 5. A total of 8,717 mortalities due to CD was recorded in 1991 out of which Southern province recorded the highest (7,895), followed by Central province with 715 deaths (Table 5). Lusaka and Copperbelt provinces recorded 83 and 24 mortalities, respectively (Table 5).

In 1992, deaths due to CD were rather lower than in 1991 (Table 2 and 5). Southern province, however, still recorded the highest mortalities (5,893) while Central province in second place recorded an increase (838). The Coppebelt province recorded 172 cases while Lusaka province had none in 1992 (Table 5). Mortalities due to ECF in 1991 were only 515 in both Northern and Eastern provinces where the disease occurs (Table 4). Eastern province recorded 414 while Northern province had only 101. There was, however, a slight increase in the number of mortalities due to ECF in 1992 with Eastern province recording 436 and Northern province recording

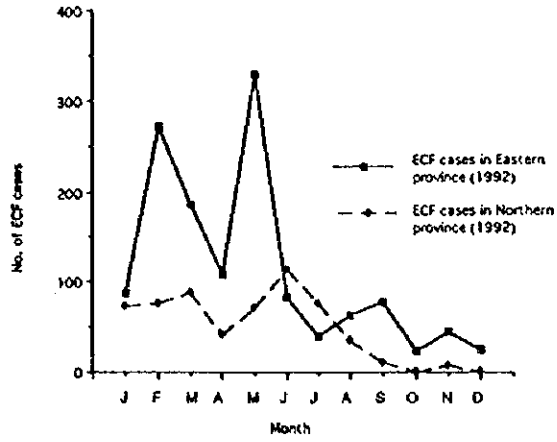

Fig. 4. The number of East Coast fever (ECF) cases in Eastern province and Northern province as recorded by Veterinary Department, Lusaka in 1992 by month.

Table 5. TOTAL NUMBER OF MORTALITIES AND CASES DUE TO CORRIDOR DISEASE IN 1991 & '92 BY PROVINCE

PROVINCE	1991		1992	
	CASES	MORTALITIES(%)	CASES	MORTALITIES(%)
SOUTHERN	15,409	7,895(51.2)	8,198	5,893(71.9)
CENTRAL	1,739	715(41.1)	6,032	838(13.9)
LUSAKA	126	83(65.9)	0	0
COPPERBELT	152	24(15.8)	364	172(47.3)
TOTAL	17,426	8,717	14,594	6,903

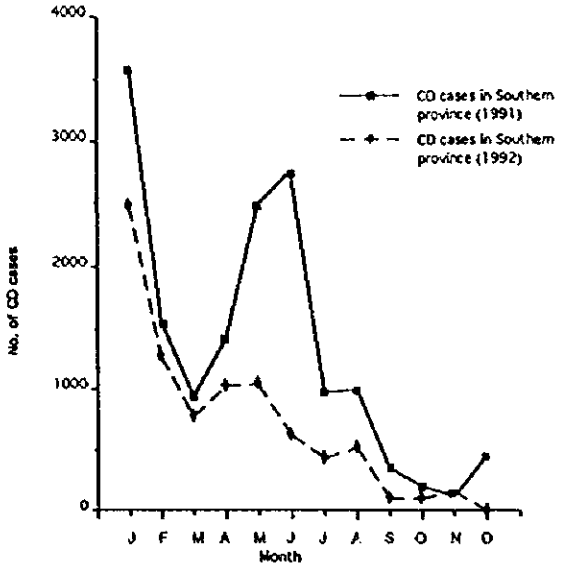

Fig. 5. The number of Corridor disease (CD) cases in Southern province as recorded by the Veterinary Department, Lusaka 1991 and 1992 by month.

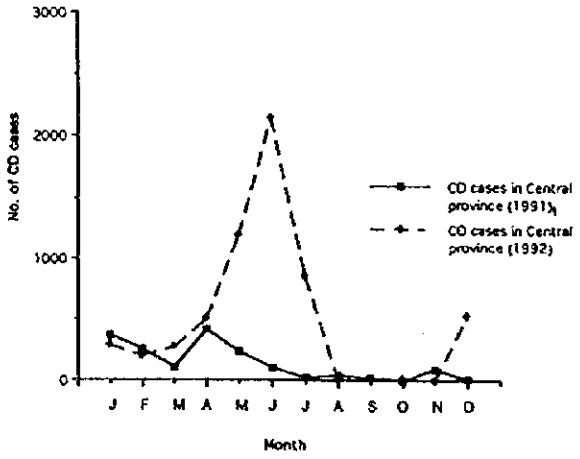

Fig. 6. The number of Corridor disease (CD) cases in Copperbelt province as recorded by the Veterinary Department, Lusaka in 1991 / 1992 by month.

225 (Table 4).

This information is derived from annual veterinary reports of the Government of the Republic of Zambia submitted to the Veterinary Headquarters in Lusaka by auxiliary veterinary personnel through their respective provincial officers. The reports provide data on disease diagnosis, treatments, recoveries and mortalities in the field. The diagnosis of theileriosis in Zambia is based on the demonstration of schizonts (Koch's blue bodies) in lymph node biopsy smears and of piroplasms in blood smears from clinically sick animals or schizonts from spleen impression smears of dead animals. At times, the diagnosis is complemented by the indirect fluorescent antibody test [3, 4] using *T. parva* antigens. Unfortunately, these personnel have very limited equipment and resources to carry out their work effectively, and therefore, the above data may only represent a fraction of the actual theileriosis situation in Zambia.

Observations

As can be seen from the figures above, the spread of ECF is some how slow in both Eastern and Northern provinces where the disease is prevalent. This could be due to fewer cattle and they are found in small isolated pockets in both provinces. Moreover, a massive immunization programme, using local *Theileria* stocks, is being carried out by the Belgian Animal Disease Project in Eastern province since 1982 [2]. The project has so far, over 50,000 calves have been successfully vaccinated.

The CD situation in the Southern province where the "Muguga cocktail", from Kenya was used to immunize cattle by the F.A.O., funded project from 1983 to 1986 is different. The initial results of this project were very encouraging [18] but later the disease spread very rapidly with high morbidity and mortality. The "Muguga cocktail" is composed of two Kenyan *T. parva parva* and one Kenyan *T. parva lawrencei* stocks. These are foreign *Theileria* strains introduced into Zambia and worse still into area where little or no information on the available local strains is available. It is known that immunized cattle, as well as those that recover naturally from *T. parva* infections or following treatment, are "carriers" of the infection and therefore, can serve as a source of infection for others [8, 9, 13, 15, 28, 33, 34].

The epidemiology of theileriosis in Zambia has become very complex. In fact one wonders whether it is still correct to refer to the disease in Southern province as CD. It is very likely that we are presently dealing with a mixed infection of *T. parva parva* and *T. parva lawrencei*. The buffalo is no longer necessary in the disease transmission cycle. Its transmission cycle is no longer restricted only to buffalo-tick-cow, but cow-tick-cow transmission has become even more frequent. It has become difficult if not impossible to distiguish CD cases from ECF. As mentioned elsewhere, it was originally possible to distinguish the two by the number of schizonts in infected lymphocytes or piroplasms in erythrocytes where-by ECF would have more and CD less. The situation nowadays is such that the number of schizonts and piroplasms in

both cases is the same, posing the question whether we are dealing with two disease complexes or just one. There is, therefore, a need to isolate and characterized *Theileria* parasites in Zambia. The next question would be whether to continue the ban on movement of cattle from Nouthern and Eastern provinces.

Other factors responsible for the rapid spread of CD in the Southern province are abundant tick-carrying wildlife; communal drinking and grazing areas; lack of adequate dipping facilities; illegal cattle movements for both social and economic purposes; development of tick resistance to acaricides and the high cost of imported curative drugs.

Control of theileriosis in Zambia

There are four main ways of controlling theileriosis in Zambia; a) vector control; b) cattle movement control; c) chemotherapy, and d) immunization.

a) Vector control

The use of acaricides to kill the tick vector still remains the most effective method of controlling tick-borne diseases in Zambia. The problem here is the high cost of acaricides. The Zambian governments policy on dipping/ spraying varies depending on the season of the year. In the rainy season, i. e. November to March, dipping/ spraying is done twice a week. Dipping/ spraying is done once very fortnight in the dry season when the tick activity/ tick numbers are less. This approach is likely to change now because theileriosis out-breaks in recent years occur almost throughout the year, especially in the last 2 years or so. This brings up the question of possible involvement of other tick-vectors such as *R. evertsi*, *R. zambeziensis* and *R. compositus*, whose role in the epidemiology of this deadly disease in Zambia requires further investigations. For example, in Zambia, *R. evertsi* is present in large numbers from March to November and is very active from July to October [16]. *R. compositus* is found on cattle in large numbers during September and October, but it could be present as early as July and as late as November and February [16].

b) Cattle movement control

Another method of theileriosis control is cattle movement restriction from theileriosis-specified areas. Movement of livestock is subject to the issuance of stock movement permits by veterinary officers. Movements within endemic areas are allowed. However, movements from endemic areas to non-endemic areas are allowed on the following conditions:

—the animals to be moved must test negative serologically by indirect immunofluorescent antibody test.

—they are treated with acaricide before they are moved to insure that they are tick-free.

—they are subjected to compulsory quarantine under close veterinary supervision.

In addition to this, if the animals are meant for slaughter, they must be branded with slaughter brands and must be slaughtered under veterinary supervision within 24 hours of their arrival at destination.

Cattle in both the Eastern and Northern provinces, where ECF appears, are not allowed to be moved to other provinces for fear of the spread of the disease. The above mentioned conditions, therefore, only apply partially in these two provinces.

c) Chemotherapy

Oxytetracyclines are effective in controlling theileriosis if given at the same time as infection as applied in the "infection and treatment method" to block both parasite and disease development. The following regimes are used to treat patent disease:

—Halofuginone (Hoechst / Rousel) Terit® 1mg/kg per os.

—Buparvaquone (Coopers / Wellcome) Butalex® 5mg/kg i.m.

—Parvaquone (Coopers / Wellcome) Clexon® 20mg/kg i.m.

Terit seems to be very active against the schizont stage while Clexon seems active against all stages of the parasite life cycle in cattle [17]. The only constraint here is the high cost of these drugs. A 40 ml bottle of Clexon, for example, costs about 25 US$. And yet this is only enough for the treatment of one adult animal since a second dose has to be given after 48 hours (one animal costs about 100 US$).

d) Immunization

So far the most prominent and widely used method of immunization against both forms of theileriosis in Zambia and elsewhere is the so called "infection and treatment method" [23]. Cattle are infected with a normally lethal dose of infective tick stabilate on day zero, followed by a single injection of a long-acting oxytetracycline to suppress both parasite and disease development [30]. This method was used by Animal Disease Control project of the Food and Agricultural Organization from 1983 to 1986 in the Southern province except foreign that *Theileria* stocks from Kenya were used. The initial results of this project were very encouraging, but later the disease spread very rapidly over-flowing beyond it's original borders. It is at the moment being used in the Eastern province by a Belgian funded project, using local *Theileria* strains, and has so far successfully vaccinated over 50,000 calves. From the low figures of theileriosis cases reported in this province, this method can be very effective as a way of controlling theileriosis so long as local *Theileria* strains are used.

Conclusion

Clearly, theileriosis is a major constraint to the development of the livestock industry in Zambia. The current strategy of relying on tick control to control the disease is becoming increasingly difficult for Zambia because of several shortcomings. Acaricides are very costly and must be bought with hard currency, a scarce commodity in Zambia. In areas heavily infested with ticks, cattle herds are walked long distances to acaricide dip-tanks or spray races as often as once every fornight for

treatment; this freqeuntly erodes land, pollutes the environment with toxic residues and may also be contributing to growing tick resistance to acaricides, in addition to over-dosing and under-dosing. It is, moreover, difficult to maintain correct acaricide strength (dipstrength) due to the short supply of water resulting in poor management of cattle diptanks. This is compounded by the country not having any form of control on the type of acaricides and pesticides being brought into the country. There is also no specific legislation regarding their safe use nor their safe disposal after use.

Curative drugs are equally expensive. For chemotherapy to be effective, the disease must be diagnosed early enough so that treatment can be given at the start of clinical disease. This is, however, difficult in *T. parva* infections because in most cases the disease becomes clinically apparent only when it has reached an advanced stage. The slow spread of theileriosis in the Eastern province proves that immunization using the infection and treatment method can be effective. This method would not only reduce losses associated with theileriosis but also reduce acaricide costs, since acaricides would then be applied strategically rather than universally. Although the immunization method of infection and treatment has been adopted in a number of countries, including Zambia, it still remains problematical for several reasons. One of the most important being "antigenic diversity". Immunity produced in an animal against one stock of the parasite may not protect against challenge with another. The second most important reason being the question of "carrier status" in immunized animals. Animals that recover naturally from *T. parva* infections or following treatment or immunization, are "carriers" of the infection and, therefore, can serve as a source of infection for others [8, 9, 33, 34]. To avoid introducing foreign strains or stocks into new areas, field immunizations against theileriosis using the infection and treatment method should be carried out using parasite populations that have been isolated from areas where immunizations are to be carried out, at least until the question of "carrier state" is cleared.

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Helminth Infections of some Domestic And Wild Animals in Zambia

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Helminthiasis is one of the major causes of impaired productivity of domestic animals and some of the helminths are important as zoonoses. Parasites of wild antelopes are of interest in connection with the veterinary importance of common species to domestic ruminants (Horak, 1978, 1981). Detailed information about helminth infection of domestic and wild animals in Zambia is scarce although survey works in this country include fascioliasis (Silangwa, 1972), helminths in the dog (Islam and Chizyuka, 1983), in goats (Islam, 1984), in horse (Islam, 1986), in domestic fowl (Islam, 1985), and in wild antelope (LeRoux, 1950).

In the present study, a total of 33 cattle, 38 sheep, 18 goats and 88 dogs originating from the Lusaka area and three impala (*Aepyceros melampus*) and two puku, (*Kobus varondi*) from Luangwa Valley were examined for helminth infestations by means of post-mortem examinations in 1986 and 1987. All the animals examined were adult.

The species identified from the cattle, sheep and goats are shown in Table 1. In the wild antelopes, *Paramphistamidae gen. sp., Cysticercus spp., Trichostrongylus falculatus, Longystrongylus meyeri* and *Trichuris globulosa* were identified both from impala and puku. *Stilesia hepatica, Haemonchus spp.* and *Oesophagostomum columbianum* from impala and *Cooperia curticei* and *Bunostomum spp.* from puku were also found. Among these species, *S. hepatica, O. columbianum* and *T. globulosa* were found in both wild and domestic animals. The infestation of *T. falculatus* in sheep has been reported in South Africa (Horak, 1981. Viljeon, 1969).

Fasciola gigantica was not found although a high prevalence in cattle from major flood plain in Southern and Western Provinces has been reported (Silangwa, 1972). This observation is presumably due to habitation of the intermediate host snail in the restricted wet flood plains or irrigation dams in Zambia. The species belonging to Anoplocephalidae, *M. benedeni. M. expansa A. centripunctata S. hepatica* and *T. giardi* were frequently detected. This is caused by throughout the year and whole day grazing on pasture in tropical savannah area. The prevalence of *Haemonchus spp.* and of *O. columbianum* were high both in sheep and goats and it is assumed that they are the primary cause of lowered productivity by parasitic gastroenteritis because of their pathogenicity and high prevalence (Allonby and Urquhart, 1972).

Stevenson *et al.* (1981) reviewed metacestodes in African game animals and suggested the small probability or non existence of humans aquiring *Taenia saginata* and *Taenia solium* from game meat. Of the present materials, *Cysti-cercus spp.* which was hookless and could not be differentiated morphologically from the cysticercus of *T. saginata* was found from the muscle samples both of impala and puku.

In dogs no trematoda were found, and most of the species identified were common parasites in tropical countries (Table 2). *M. lineartus, S. lupi* and *P. affinis* which require second intermediate hosts or possibly take paratenic hosts, like small wild mammals or reptiles were frequently identified, showing the important role of wild animals in parasite prevalence in this area. There is no earlier report of *P. affinis* in Zambia in spite of high prevalence in the present survey.

All the specimens examined have been deposited in the parasitological collection of the School of Veterinary Medicine, University of Zambia.

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Table 1 Prevalence of Helminths in 33 cattle, 38 sheep and 18 goats.

	Number (%) of positive animals		
Species	Cattle	Sheep	Goat
Paramphistomatidae gen. spp.	5 (15.2)	7 (12.1)	0
Schistosoma spp.	4 (12.1)	0	0
Moniezia benedeni	2 (6.1)	0	0
Moniezia expansa	0	8 (21.1)	0
Thysanezia giardi	0	0	3 (16.7)
Avitellina centripunctata	0	8 (21.1)	0
Stilesia hepatica	0	7 (18.4)	3 (16.7)
Taenia hydatigena (cysticercus)	0	1 (2.6)	7 (38.9)
Taenia multiceps (coenurus)	0	0	1 (5.6)
Strongyloides papillosus	0	0	1 (5.6)
Haemonchus spp.	7 (21.2)	27 (71.0)	11 (61.1)
Trichostrongylus colubriformis	0	0	5 (27.8)
Cooperia oncophora	1 (3.0)	0	0
Cooperia punctata	4 (12.1)	1 (2.6)	0
Cooperia pectinata	5 (15.2)	4 (10.5)	0
Oesophagostomum columbianum	0	3 (7.9)	9 (50.0)
Bunostomum phlebotomum	3 (9.1)	0	0
Skrjabinema ovis	0	0	1 (5.6)
Neoascaris vitulorum	1 (3.0)	0	0
Setaria labiatopapillosa	4 (12.1)	0	0
Trichuris discolour	3 (9.1)	9 (23.7)	0
Trichuris globulosa	0	1 (2.6)	0
Trichuris skrjabini	0	2 (5.2)	0

Table 2 Prevalence of helminths in 88 dogs.

Species	Number (%) of positive dogs
Mesocestoides lineatus	4 (4.5)
Taenia hydatigena	1 (1.1)
Dipylidium caninum	33 (37.5)
Toxocara canis	14 (15.9)
Ancylostoma caninum	54 (61.4)
Ancylostoma braziliense	3 (3.4)
Pterigodermatites affinis	11 (12.5)
Spirocerca lupi	5 (5.7)

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PRELIMINARY EFFICACY TRIAL OF CYMELARSAN IN MICE ARTIFICIALLY INFECTED WITH *TRYPANOSOMA BRUCEI BRUCEI* ISOLATED FROM A DOG IN ZAMBIA

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ABSTRACT

An efficacy trial of Cymelarsan® on a Zambian strain of *Trypanosoma brucei brucei* was done. Twenty-five male mice were infected intraperitoneally with 10^6 of *T. b. brucei* isolated from a dog. Five groups of 5 mice were treated with 0 (control), 0.25, 0.5, 1.0 and 2.0 mg/kg Cymelarsan, respectively. The target was to achieve aparasitaemia for 30 days post-treatment, euthanising those that remained parasitaemic or relapsed before then. The 0.25 and 0.5 mg/kg groups remained parasitaemic although the parasitaemic levels were reduced. The 1.0 mg/kg group had a proportion of aparasitaemic mice. However, all mice in the 2.0 mg/kg group remained aparasitaemic until day 20 when 2 mice relapsed. These resullts suggested that more than 2.0 mg/kg was required to eliminate this strain.

Key Words: Trypanosomiasis, Cymelarsan (Mel Cy), mice

The control of trypanosomiasis in prevalent countries has primarily depended upon the effective use of chemotherapy. Vaccination, which to date has attracted a lot of active research, has not been very successful due to the parasite's ability to vary its antigenic coat[5]. Furthemore, over a long period of time very few drugs have been developed against the parasite, resulting in strains resistant to the few drugs in use. *Trypanosoma brucei brucei (T. b. brucei)* is one of the common trypanosoma species in Zambia. The most commonly used drugs to control this and other trypanosome species in the country have mainly been diminazene aceturate (Berenil® ; Hoechst, S. Africa), and isometamidium chloride (Samorin® ; M&B, S. Africa). Although information on the levels of strains resistant to these drugs is limited in Zambia, the reported

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levels worldwide have indicated the need to search for alternative drugs.

Cymelarsan (Mel Cy. RM110; Rhone Merieux, France) was developed in 1985 specifically for veterinary use by Dr Friedheim from a combination of melarsenoxyde and cysteamine[9]. The drug, which is a trivalent arsenical and is currently being evaluated as a trypanocide, has been shown to have activity against the *Trypanozoon* subgenus to which *T. b. brucei* belongs in artificial and natural infections of camels, horses, cattle and buffaloes[2]. In camels artificially infected with *T. b. evansi* in Niger, the parasite disappeared from the blood for 63 days after treatment at dosages of 0.625–1.25 mg/kg[12]. Similar results were reported from Ethiopia in artificially infected camels[13]. In Kenya, however, of the naturally infected camels that were treated at dosages between 0.2–1.2 mg/kg one of them treated at 0.4 mg/kg relapsed[8]. It is not reported whether this was a case of re-infection or a genuine relapse of the original infection. The drug is available as a white powder highly soluble in water. It is administered either intramuscularly or subcutaneously.

The present study was carried out to observe the effect of Cymelarsan on a local (Zambian) strain of *T. b. brucei* and establish a dosage appropriate to treat cases for up to 30 days post infection (PI). Research to date has concertrated on *T. evansi* and little information is available on the other species in the *Trypanozoon* subgenus and the animal species they infect. This study also investigates these factors. The strain of *T. b. brucei* used was isolated from a dog in Chipata, the eastern province of Zambia and is infective to mice. The strain has been tested and found to be susceptible to Berenil at the normal curative dose of 3.5 mg/kg[6].

Twenty-five in-bred, white male mice weighing an average of 25 grams were used in the experiment. Mice were infected with *T. b. brucei* by inoculation of approximately 10^5 parasites intraperitoneally (I. P.). The mice were monitored for development of infection before treatment on days 3 and 8. They were then divided into treatment groups A, B, C, D and E, with A being the control while B, C, D and E received 0.25, 0.5, 1.0 and 2.0 mg/kg I. P. of Cymelarsan, respectively. This dosage range was arrived at from previous knowledge on an experiment carried out on *T. b. evansi* in which 2 mg/kg was the maximum dose required to cure early and chronic infection in mice[11]. After treatment, persistence of infection and/or relapse was monitored on days 1, 3, 6, 13, 16 and 20. It was intended to examine the mice twice weekly for 30 days post-treatment sacrificing those groups that tested positive. The dosage was certified curative if it caused aparasitaemia for 30 or more days. The parasitaemic levels before and after treatment were determined by direct wet blood film examination under a microscope at ×400 magnification[7]. These levels were recorded on the basis of log equivalent values (L. E. Vs) using the Lumsden matching technique.

The L. E. Vs of days 3 and 8 before treatment were on the average of 8.4 in all groups of mice. After treatment with Cymelarsan the 2 mg/kg group remained

aparasitaemic up to day 20 when two mice in the group tested positive and the whole group was euthanised. Aparasitaemia in the 1 and 2 mg/kg groups was noticed as early as 24 hours after treatment. The L. E. Vs in the 0.25 mg/kg group decreased after treatment but parasitaemia never disappeared completely, whereas the 0.5 and 1 mg/kg groups had a proportion of mice negative to infection up to day 16 when all groups showing infection were euthanised. The exact details of results in all groups after treatment are summarised in Table 1:

From the experiment, it was apparent that either a reduction in the levels of parasitaemia or complete aparasitaemia, indicating some form of drug effect, was obtained after administration of the drug. However, whether this was a trypanocidal or merely a transient suppressive effect could not conclusively be deduced from this experiment. It was also apparent from the experiment, as a result of the relapse observed after 20 days in the 2 mg/kg group, that to cure completely or suppress the parasite for 30 days a dose higher than 2 mg/kg was required. This differs from the minimum curative dose (M. C. D) required by mice infected with *T. b. evansi* shown in an earlier experiment[1)], although the significance of this difference was not statistically tested. That the species difference was responsible for this observed difference is possible, however, the possibility of this local strain responding differently should equally be considered. An earlier report[3)] in which *T. b. brucei* and *T. b. evansi* were tried together did not indicate the exact dose used to cure the infection of *T. b. brucei*, although it reported a similarlity in the requirement of a higher dose of the drug to cure chronic infections of both species. Therefore, to establish whether the difference in M. C. D between *T. b. brucei* and *T. b. evansi* is a result of species difference or merely a characteristic of this strain, more experiments should be carried out.

Another factor that could have influenced the higher dose requirement might have been the length of the postinfection period when treatment was effected. This is

Table 1. The number of aparasitaemic mice and the duration of aparasitaemia per group of mice exposed to different dosages of Cymelarsan.

Group	No.	Dosage mg/kg	Day :	No. of aparasitaemic mice per group 1	3	6	13	16	20
A *	5	0.0		0/5	0/5	0/5	0/5	0/5	0/5
B	5	0.25		0/5	0/5	1/5	0/5	0/5	—
C	5	0.5		1/5	4/5	5/5	0/5	1/5	—
D	5	1.0		4/5	4/5	5/5	2/5	2/5	—
E	5	2.0		5/5	5/5	5/5	5/5	5/5	3/5

* = Untreated control.
No. = Number of mice.
— = Euthanised.

especially so with *T. b. brucei*, which localises in the brain, a privileged site, where it is inaccessible to drugs or the concentration of the drug that penetrates the tissue is insufficient to be trypanocidal[4,5,10]. *T. b. brucei* being a cause of acute infections in mice, 8 days PI was a long enough period for it to establish itself in tissues and thus interfere with the M. C. D. This could further explain the relapses noticed in the 2 mg/kg group after 20 days as being relapses from the tissues. However, to what extent Cymelarsan is effective on tissue forms, if any, is a point that requires investigation.

Berenil® , to which Cymelarsan is known to exhibit cross-resistance[1], was found to cure this parasite at the normal dose of 3.5 mg/kg[6]. It is therefore, not possible that cross-resistance was responsible for the higher than normal dose required to treat the infection. Furthermore, Cymelarsan has not been used in this region before so direct resistance does not seem a credible reason either.

More research on the drug's availability, applicability and affordability for general practice would be especially valuable as the diseases it is meant to treat are prevalent in third world nations where money is an important limiting factor in the choice and use of drugs.

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Effect of Berenil on the Kinetoplast of *Trypanosoma gambiense* Pararosaniline Sensitive and Resistant Clone in Mice

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Abstract

After the inoculation of Berenil into *Trypanosoma gambiense* infected mice, dyskinetoplastic forms appeared as a result of inhibition of kinetoplast duplication without any affect on nuclear and cytoplasmic duplication. The present study demonstrated that in some dividing forms with a nucleus appeared after the treatment with Berenil a flagellum in which the kinetoplast can not be seen near to it, was observed at the posterior end of the parasite and one kinetoplast with or without a flagellum migrated anteriorly. That is, a dividing form containing one kinetoplast, one nucleus and two flagella (1K1N2F), and a dividing form containing one kinetoplast, one nucleus and a flagellum (1K1N1F) were observed. In the latter, the kinetoplast without a flagellum migrated close to the nucleus or anteriorly far from it. Such trypanosomes appeared in the original clone after the treatment, but, not in the *p*-rosaniline resistant clone. We have never seen such migration of the kinetoplast without a flagellum in trypanosomes treated with various chemicals except for Berenil in the present study. The present study also indicated that the original clone was more sensitive than the *p*-rosaniline resistant clone to the effect of Berenil in inhibiting kinetoplast division.

Key words: Berenil, dyskinetoplastic form, kinetoplast migration, *Trypanosoma gambiense*

Introduction

Many chemicals interacting with DNA, such as acriflavine, pararosaniline, ethidium bromide, hydroxystilbamidine and furazolidon are known to be trypanocidal and to induce dyskinetoplastic trypanosomes and the deformation in the ultrastructure of the kinetoplast (Cavalieri and Angelos, 1950; Inoki, 1956; Neville and Davies, 1966; Le Pecq and Paoletti, 1967; Ono and Inoki, 1971, 1973 and 1975). These chemicals have no effect on nuclear and cell division at the concentrations that induce dyskinetoplastic forms.

Berenil (Diminazene aceturate, Hoechst, Germany) shows a preferential binding to kinetoplast DNA and inhibits DNA synthesis in *Trypanosoma mega* (Newton and Le Page, 1967). Therefore, when Berenil was inoculated into animals infected with various *Trypanosoma* species, *T. evansi*, *T. equiperdum*, *T. congolense* and *T. brucei*, dyskinetoplastic forms appeared in the peripheral blood (Killick-Kendrick, 1964: Riou and Benard, 1980: Chitambo et al., 1992). But, analysis of formation of dyskinetoplastic forms has not been attempted. Chemicals inducing dyskinetoplastic forms, such as *p*-rosaniline, hydroxystilbamidine and Berenil do not intercalate into DNA, but show a preferential binding to kinetoplast DNA. Therefore, there may be the interaction between effect of *p*-rosaniline and one of Berenil on trypanosomes. In the present studies, we examined morphologically the mode of formation of dyskinetoplastic forms and a difference of effect on these clones after injection of Berenil into mice infected with *T. gambiense* of *p*-rosaniline sensitive original and resistant clones.

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Materials and Methods

Trypanosoma sp.

The Wellcome strain of *Trypanosoma gambiense*

was used in the present study. The strain was derived from Dr. H. Takayanagi in 1989 (Department of Medical Zoology, School of Medicine, Nagoya City University). Since then it has been maintained in our laboratory by serial passages in mice and preserved by 10% DMSO in 0.01 M PBS (pH 7.2) in liquid nitrogen. Two clones of the Wellcome strain were used in the present study. One was the original clone (hereafter WS) and the other was the clone WR isolated from WS treated with 50 μg *p*-rosaniline per g mouse body weight. WS was sensitive to *p*-rosaniline, ethidium bromide and acriflavine, eliciting about 25% of dyskinetoplastic form (AK) 4 hr after intraperitoneal injection with these chemicals into infected mouse. WR which can still grow after injection of as much as 50 μg *p*-rosaniline/g mouse body weight, do not produce dyskinetoplastic trypanosomes even by a dose of 10 μg *p*-rosaniline/g body weight. WR was obtained from WS repeatedly treated with *p*-rosaniline (Inoki and Matsushiro, 1959). The WR line has been maintained by serial passages in ICR mice and preserved by 10% DMSO in 0.01 M PBS in liquid nitrogen for more than three years. Division of trypanosomes begins in the basal body. And then, it is followed by binary fission of the kinetoplast, and later by division of the nucleus and cytoplasm. Therefore, trypanosomes of WS and WR before the treatment with Berenil are undivided forms with one kinetoplast and one nucleus (1K1N), dividing forms with two kinetoplast and one nucleus (2K1N) and dividing forms with two kinetoplasts and two nuclei (2K2N). ICR mice weighing approximately 30 g were used in the present study. The mice were kept in the conventional condition.

Infection of mice, treatment and preparation of blood smears

When trypanosomes had reached a level of 5×10^8 trypanosomes/ml blood stream in ICR mice 3 days after intraperitoneal inoculation with approximately 1×10^5 trypanosomes, the mice were injected intraperitoneally with 10 μg/g of Berenil. Blood samples were taken at 1, 2, 3, 4 and 8 h after treatment. They were stained with Giemsa after hydrolysis with 1N HCl at 60°C for 2 min and examined under a light microscope. The numbers of trypanosomes of various forms were counted on 1,000 randomly selected parasites in each stained blood smear. All values were given as means± standard deviation of six replicate experiments.

Results

For analysis of effect of Berenil on WS and WR, the numbers of appearance of trypanosomes of various forms were examined at intervals after injection of 10 μg/g of Berenil into infected mice (Table 1). Figures 1 and 2 show dividing forms of untreated trypanosomes. When two kinetoplasts in 2K1N separated, a flagellum is always observed near to each kinetoplast (Fig. 1). Two kinetoplasts in 2K2N are seen posteriorly from two nuclei (Fig. 2). After the treatment with Berenil, however, trypanosomes of various forms appeared as shown in Figs. 3–6. In Table 1, the rate of appearance of trypanosomes of various forms is almost the same in both clones before the treatment with Berenil. The number of 2K1N in WS decreased greatly with the lapse of time after the treatment with Berenil, while that in WR till 4 hr did not decrease so much as in WS. The number of 2K2N also decreased in WS, but no decrease in number of this type are seen by 4 hr in WR. Dividing form with one kinetoplast and two nuclei (1K2N) are seldom produced in both clones before the treatment. But, the number of this type in WS increased 2 hr after the treatment. The increase in WR by 4 hr was not so much as in WS. Dyskinetoplastic form without the kinetoplast (Fig. 3) increased 1 hr in WS after the treatment and then increased remarkably with the lapse of time. While no increase was observed in WR by 4 hr after the treatment. The decrease in the numbers of 2K1N and 2K2N and the increase in those of 1K2N and of dyskinetoplastic forms which were demonstrated by 2 hr in WS, were observed 8 hr in WR after the treatment. The number of dyskinetoplastic forms in WS was greater than in WR. Anucleate forms with one kinetoplast (1KAN) were a few observed in both clones.

In some dividing forms with one nucleus appeared after the treatment with Berenil, one flagellum in which the kinetoplast can not be seen near to it, was observed at the posterior end of the parasite and one kinetoplast with or without a flagellum migrated anteriorly. That is, 1K1N with two flagella (1K1N2F, Fig. 4) and 1K1N with one flagellum (1K1N1F)

Table 1 Changes in the numbers of trypanosomes of various forms* appeared after injection of 10 μg/g Berenil into mice infected with *T. gambiense* of *p*-rosaniline sensitive and resistant clones

Forms	Hours after Berenil treatment					
	0	1	2	3	4	8
1K1N†	810.1 ± 38.2#	828.1 ± 33.5	789.8 ± 19.9	712.1 ± 42.6	658.6 ± 49.5	622.8 ± 59.1*
	808.8 ± 18.4	839.5 ± 25.9	855.5 ± 42.8	837.2 ± 20.4	825.7 ± 23.1	739.8 ± 24.7*
2K1N‡	113.0 ± 21.9	74.1 ± 17.1	31.3 ± 10.5	25.8 ± 11.6	26.0 ± 13.1	28.0 ± 12.6
	116.0 ± 14.0	88.8 ± 18.4	83.6 ± 27.6	75.1 ± 10.6	54.1 ± 12.4	18.6 ± 10.1
2K2N§	64.5 ± 19.2	63.3 ± 16.6	36.0 ± 14.9	28.8 ± 10.4	30.3 ± 15.6	17.1 ± 6.5
	59.0 ± 6.4	59.3 ± 12.5	42.6 ± 16.3	49.1 ± 16.4	51.2 ± 9.3	20.6 ± 7.3
1K2N‖	0.8 ± 1.3	2.1 ± 1.9	16.0 ± 8.2	40.8 ± 16.2	43.1 ± 7.8	7.5 ± 5.2
	0.3 ± 0.5	0.6 ± 1.2	1.1 ± 1.1	4.5 ± 3.9	10.2 ± 5.7	46.8 ± 12.3
AK1N¶	8.8 ± 3.5	18.3 ± 4.2	93.0 ± 11.4	147.3 ± 18.6	179.5 ± 45.8	263.1 ± 54.8
	16.0 ± 4.3	10.0 ± 4.7	12.1 ± 4.7	16.8 ± 4.2	22.1 ± 7.3	162.8 ± 15.2
AK2N**	0.5 ± 0.8	0.5 ± 0.5	1.0 ± 0.6	1.3 ± 1.0	1.5 ± 1.8	9.1 ± 7.2
	0.3 ± 0.8	0.3 ± 0.5	0.3 ± 0.5	0	0.1 ± 0.3	2.5 ± 2.0
1K1N1F††	0.3 ± 0.8	4.1 ± 2.3	12.1 ± 6.1	10.6 ± 3.5	8.3 ± 3.0	48.6 ± 14.9
	0.2 ± 0.4	0	0	0.1 ± 0.3	0	1.5 ± 1.2
1K1N2F§§	0.3 ± 0.8	3.0 ± 2.3	19.0 ± 8.2	47.6 ± 25.9	53.3 ± 21.5	7.6 ± 3.0
	0	1.0 ± 2.0	2.5 ± 2.1	9.3 ± 7.8	33.4 ± 14.3	8.3 ± 4.1
1KAN◇	0.2 ± 0.4	0.2 ± 0.4	0	0	0	0.6 ± 1.0
	0	0	0	0.1 ± 0.3	0.2 ± 0.4	1.6 ± 1.9

*The numbers of trypanosomes of various forms are counted on 1,000 randomly selected parasites. †Undividing form with one kinetoplast and one nucleus. ‡Dividing form with two kinetoplasts and one nucleus. §Dividing form with two kinetoplasts and two nuclei. ‖Dividing form with one kinetoplast and two nuclei. ¶Dyskinetoplastic form with one nucleus. **Dyskinetoplastic form with two nuclei. ††Dividing form with one nucleus and one kinetoplast in which a flagellum is not observed near to it. §§Dividing form with one nucleus and one kinetoplast in which a flagellum is observed near to it. ◇Anucleate form with one kinetoplast.

#All values are given as means ± standard deviation of six replicate experiments.

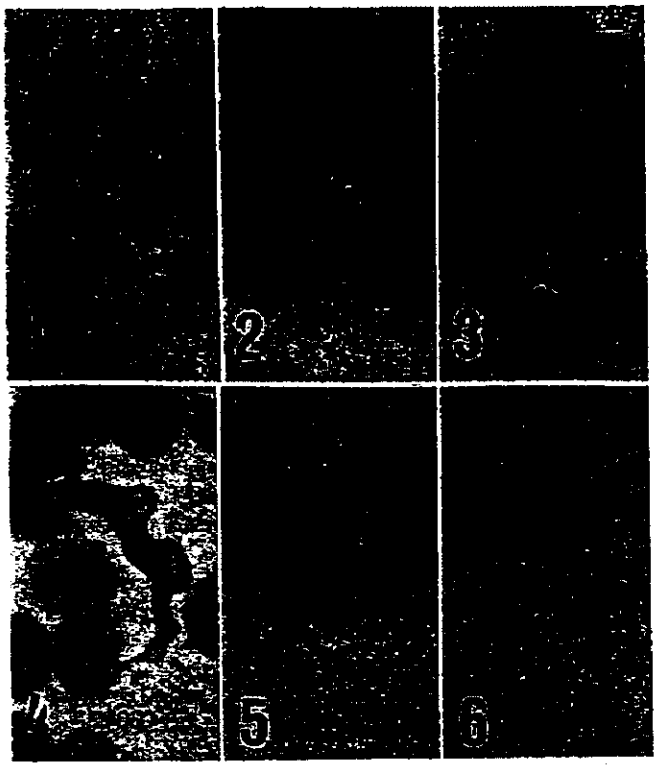
☆ The upper line in each form corresponds to WS. ★ The lower line corresponds to WR.

were observed. Such dividing forms increased with the lapse of the time. In 1K1N1F, the kinetoplast without a flagellum was seen close to the nucleus (Fig. 5) or anteriorly far from it (Fig. 6). These forms increased in WS after the treatment, but, not in WR.

Discussion

We have demonstrated that in *T. gambiense* treated with many kinds of dyskinetoplastic form inducing substances dyskinetoplastic forms were produced by the selective inhibition of kinetoplast duplication without any affect on nuclear and cytoplasmic duplication (Inoki 1956; Ono and Inoki 1971, 1973, 1975).

In the present study, the total number of trypanosomes having two nuclei, such as 1K2N, 2K2N and AK2N within 4 hr in WS and 8 hr in WR after treatment with Berenil was about the same as the number of trypanosomes having two nuclei before the treatment in these clones. This finding suggests that Berenil resulted in the production of



Figs. 1 and 2 Lightmicrographs showing a dividing form of WS untreated with Berenil. ×1,600

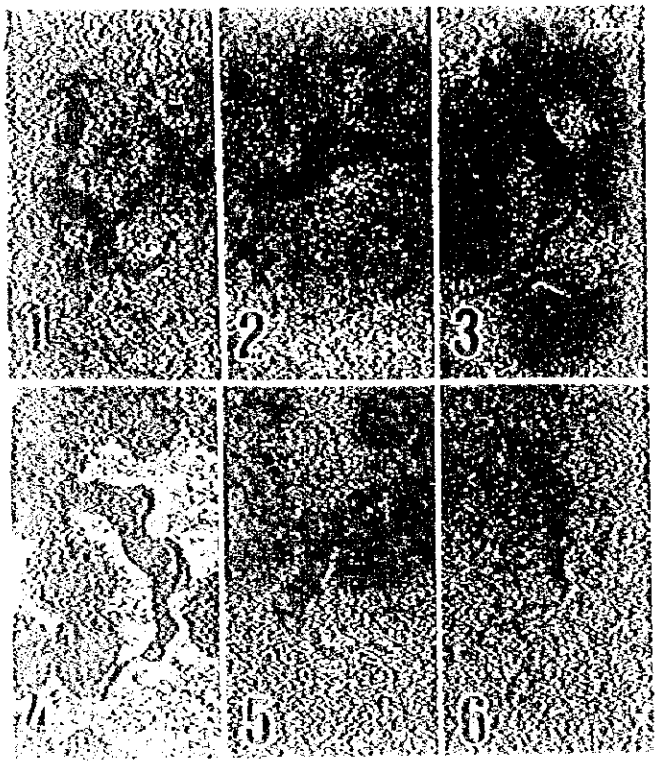
Figs. 3–6 Lightmicrographs showing trypanosomes obtained 4 hrs after injection of 10 μg/g of Berenil into mice infected with WS (all Figs. except for Fig. 4) or WR (Fig. 4). ×1,600

Figs. 1 and 2 When two kinetoplasts separated, a flagellum is already observed near to each kinetoplast. Two kinetoplasts are seen posteriorly from two nuclei.

Fig. 3 Dyskinetoplastic form. Figs. 4–6. Trypanosomes with one kinetoplast and one nucleus with two flagella (Fig. 4), and with one kinetoplast and one nucleus with a flagellum are observed. In the latter, the kinetoplast is seen close to the nucleus (Fig. 5) or anteriorly far from it (Fig. 6).

dyskinetoplastic forms without any affect on nuclear division. In 8 hr after the treatment, the total number of trypanosomes having two nuclei in WS was less than half of that in WR. It means that the nuclear division is inhibited in WS 8 hr after the treatment, but, not in WR.

The present study reported that in IKINIF appeared after the treatment with Berenil in WS, one kinetoplast without a flagellum was observed close to the nucleus or anteriorly far from it. In trypanosome replication, the daughter basal bodies separate before the kinetoplast, which has just divided, begins to separate, and then the nucleus and cytoplasm divide successively. Therefore, disorder in formation of basal body due to inhibition or delay of kinetoplast division might result in appearance of trypanosomes having the kinetoplast without a flagellum. *p*-rosaniline, hydroxystilbamidine and Berenil do not intercalate, but show a preferential binding to kinetoplast DNA and produce the dyskinetoplastic forms. Therefore, there may be a similarity in the effect of these chemicals on trypanosomes. However, we have never seen such migration of kinetoplast without a flagellum in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trypanosomes treated with various chemicals including hydroxystilbamidine and *p*-rosaniline and except for Berenil in the present study (Ono and Inoki, 1971, 1973, 1974, 1975; Ono and Nakabayashi, 1978, 1979, 1980, 1987), suggesting that the action mechanism of Berenil differs at least in a part from those of other chemicals.

The present study indicates the existence of some relationship between the actions of Berenil and *p*-rosaniline on the kinetoplast of trypanosomes. WR which obtained from WS by repeated treatment with *p*-rosaniline without exposure to Berenil, was more resistant than WS to the effect of Berenil in inhibiting kinetoplast division and in inducing disorganization and abnormality of kinetoplasts. IKINIF showing migration of kinetoplast without the flagellum appeared in WS after the treatment with Berenil, but, not in WR, indicating that an organization of kinetoplast duplication in WR can operate to some extent even under the existence of Berenil. The chemicals also resulted in inhibition of nuclear division in WS 8 hr after the treatment, but, not in WR, suggesting the existence of a relationship between the actions of Berenil and *p*-rosaniline on the nucleus as well as the kinetoplast of trypanosomes. Studies on the effect of DNA synthesis inhibitor and DNA interacting chemicals on trypanosome is useful to clarify differences in the mechanism of DNA synthesis in the nucleus and the kinetoplast of trypanosomes.

It is interesting to examine whether dyskinetoplastic forms appeared after the treatment with berenil are able to multiply or not. Treatment of infected mice with 10 μg Berenil/g body weight induced the high percentage of the dyskinetoplastic forms and caused a temporary clearance of parasite followed by relapsing parasitemia. Although not shown in the data, the percentage of dyskinetoplastic forms in relapsing mice always returned to the initial level prior to the treatment with Berenil. After the treatment of infected mice with Berenil, the selection of kinetoplastic forms as a result of the extinction of dyskinetoplastic forms and the conversion from dyskinetoplastic form to kinetoplastic form due to restoration of the kinetoplast DNA might occur in the latent period in which trypanosomes can not be seen in the peripheral blood. Therefore, the further studies are necessary to clarify multiplicability of dyskinetoplastic form appeared after the treatment with Berenil.

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THE FIRST REPORTED CASE OF CANINE VISCERAL LEISHMANIASIS IN ZAMBIA.

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ABSTRACT

Leshmaniasis is caused by the genus *Leishmania* that affects various mammals , but most commonly humans and dogs. Cutaneous and visceral forms are recognized. The visceral form is chronic and often fatal. *Leishmania* species. are widely distributed in Central and South America, East and West Africa, Asia. the Mediterranean countries. but rare in southern Africa, and canine cases have not been reported in Zambia.

A male 12 year old dog had been kept in Lusaka for two years. The owner used horticultural chemicals and the dog was drinking the contaminated water. The dog was brought to the clinic on the 5th of September, 1994 because of anorexia. polydypsia and occular discharge. The dog was in good condition apparently. but showed tachycardia. weak pulse and icterus. Haematology : PCV 29%, RBC 3.70 x 10^6/micro ml, WBC 14.6 x 10^6/micro ml. nuetrophils 89.6%. lymphocytes 7.5% , eosinophils 0.9%, monocytes 1.8% basophils 0.2% Hb 9.2 g/dl. total protein 8.0g/dl, serum protein 6.3 g/dl, GOT 139.7 IU/L. GPT 43.1 IU/L, alkaline phosphotase 379.5 IU/L, GGT 13.9 IU/L, BUN 55.7 mg/dl and creatinine 5.1 mg/dl.

The dog died the following day and was autopsied. Microscopically. medium splenomegaly, yellowish degenerated liver with jaudince. scattered neoplastic lesions in the kidney and extremely round heart were found.

Histologically. in the liver hepatocytes were diffusely fatty-changed and macrophages, in which many granular bodies i.e amastigotes were included. scattered diffusely or as smallest nodule and focal monocyte-infiltrating lesions. In the spleen. amastigote-laden macrophages proliferated diffusely or nodularly. In the lung. the macrophages scattered in the alveolar wall. In the heart, there were monocytic small lesions and fibrinoid degeneration of the arterial wall was found. In the kidney there were focal lymphocytic lesions. Amastigote-laden macrophages were scarce in the heart and kidney lesions. The case was diagnosed as Visceral Leshmaniasis. The dog was apparently in chronic chemical intoxication. which must have induced *Leshmania* infection . It was not known were the dog could have aquired the infection. but the dog had been in Lusaka for two years at the time of presentation. It is possible that the dog was a carrier of the Leshmania parasite.

* Full paper not received by time of production of proceedings.

Characterization of Some *Theileria parva* Stocks from Zambia Using Monoclonal Antibodies

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ABSTRACT. *Theileria parva* parasites have been isolated from different locations in Zambia where malignant theileriosis has been recorded. A total of 16 bovine lymphocytic cell lines infected with *T. parva* schizonts were characterized using a panel of anti-schizont monoclonal antibodies (MAbs). Comparison of the *Theileria* stocks isolated before (old) and after (new) the Muguga cocktail of *T. parva* from Kenya was used to vaccinate cattle against theileriosis in Zambia revealed differences in their reactivity against MAbs. The new isolates are showing MAb profiles similar to that exhibited by the Muguga cocktail which was used to vaccinate cattle in these areas between 1983 and 1989. These results suggest that the use of the Muguga cocktail to vaccinate animals against theileriosis in Zambia may have introduced *Theileria* stocks of different antigenic properties. — KEY WORDS: immunization, monoclonal antibody, Muguga cocktail, *Theileria parva*.

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Two *Theileria* parasites, *T. annulata* and *T. parva* cause severe clinical disease in cattle in Africa, Asia and the Middle East. The most economically important species in Africa is *Theileria parva*. *T. parva* parasites cause diseases that are of considerable economic importance in Zambia and other countries in East, Central and Southern Africa. In Zambia, theileriosis manifests itself in two forms namely Corridor disease (CD) and East Coast fever (ECF). CD appears in Southern, Central, Lusaka and Copperbelt provinces while ECF is found in the Northern and Eastern provinces (Fig. 1). Cattle can be immunized against ECF and CD by an infection and treatment method [9, 19]. However, immunity induced using a particular *T. parva* stock may not necessarily confer protection against challenge with other *T. parva* stocks [6, 19, 20]. This fact makes it necessary to characterize the existing *Theileria* parasites from areas where this disease is prevalent before choosing a particular stock for use in the infection and treatment method. Several researchers have attempted to characterize *Theileria* parva stocks using *in vitro* methods such as monoclonal antibody (MAb) profiles [15] and the polymerase chain reaction (PCR) amplification of Tpr I repetitive DNA sequences [1]. Here we used *T. parva* anti-schizont MAbs obtained from the International Livestock Research Institute (ILRI) to characterize some *T. parva* stocks obtained from different locations in Zambia after the "Muguga cocktail" was used to vaccinate cattle against theileriosis in Southern and Central provinces of Zambia. The Muguga cocktail is a concoction of 2 Kenyan bovine *Theileria* stocks and a buffalo-derived *Theileria* stock from Tanzania passaged in cattle several times in Kenya. The old Zambian stocks were isolated by the Food and Agricultural Organization (FAO) between 1982 and 1986 and by the Belgian Animal Disease Control Project (ASVEZA) between 1985 and 1989. We then compared the MAb profiles of the new isolates obtained after 1994 with those of the old Zambian *Theileria* stocks which were isolated before the cocktail was used . All the old Zambian

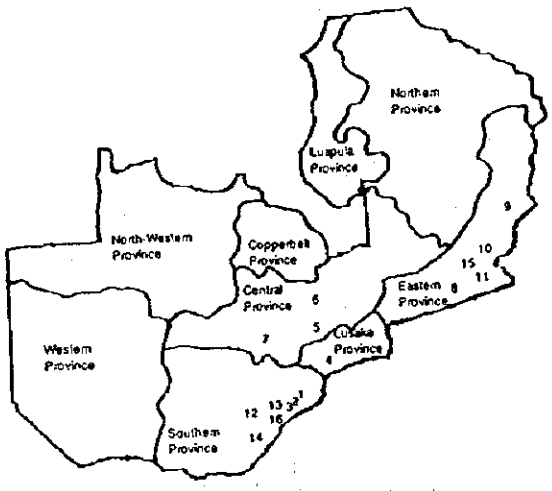

Fig. 1. Map of Zambia showing the 9 provinces. The numbers indicate the locations where the parasites used in the study were isolated. New isolates: 1–7, Old isolates: 8–16. 1. Magoye, 2. Sikabenga, 3. Ufwenuka, 4. Lusaka, 5. Chisamba, 6. Kabwe, 7. Mumbwa, 8. Katete, 9. Lundazi, 10. Langa, 11. Genda, 12. Zambia 3, 13. Zambia 5, 14. Zambia 23, 15. Zambia 2, 16. Zambia 22.

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Theileria isolates were previously characterized by ILRI.

MATERIALS AND METHODS

Cattle: The cattle used were either experimentally infected in the laboratory or were field clinical cases. The animals which were used for experimental infections were bought from farms with no history of over theileriosis. They were in addition screened for *Theileria* antibodies and only those found negative were used.

Origin of Theileria parva cultures: Old *T. parva* stocks were obtained from the Belgian project designated Lundazi, Katete, Langa, Genda, Zambia 2, Zambia 3, Zambia 5, Zambia 22 and Zambia 23 (Fig. 2). The Zambia 2, 3, 5, 22, and 23 were provided in the form of cell lines in culture while the Katete, Lundazi, Langa and Genda stocks were supplied as stabilate forms and therefore had to be passaged through clean calves from which cell lines were established [3]. Some of the new *T. parva* stocks were isolated from infected cattle in the field and were named according to their place of origin (nearest town, village or farm) while others were isolated from experimentally infected cattle in the laboratory as described by Cunningham *et al.* [5]. *Theileria* infected lymphoblastoid cell cultures were then established with infected lymphoid cells from the blood and lymph node samples from the infected cattle by the method of Brown [3]. Table 1 shows the old and new Zambian *Theileria* stocks used in this study.

Source of monoclonal antibodies: MAbs were obtained from ILRI, Nairobi, Kenya. They were macroschizont-based. MAbs 1, 2, 3, 4 and 7 were derived from mice inoculated with *T. parva* (Muguga)-infected bovine lymphocytes [18]. MAbs 10 and 12 were raised against *T. parva* (Kiambu 5), while 15 was raised against *T. parva* Marikebuni [15]. MAb 20 was raised from mice inoculated with lymphocytes infected with a buffalo-derived *Theileria* parasite [17, 18].

Preparation of antigen: Antigen slides were prepared from cultures infected with schizonts by a method similar to that described by Goddeeris *et al.* [10]. Briefly, two hundred milliliters of cell culture containing approximately 2×10^6 cells were centrifuged at 200 g for 10 min, and the cells were washed twice, and cell counts and viability were determined in 1% trypan blue solution. The percentage of schizont-infected cells was determined at the time when the antigen smears were being prepared. It was found that 75–85% of the cells were infected with *Theileria* schizonts as determined by microscopic observation of Giemsa stained smears. An equal volume of fixative (3.7% formaldehyde in phosphate-buffered saline, PBS at pH 7.2) was added drop by drop with gentle stirring. Cells were then centrifuged at 200 g for 5 min, washed 3 times, resuspended in 2 *ml* of PBS, and adjusted to give 1×10^6 cells per *ml*. Cells were distributed onto Wellcome PTFE Multispot slides (C. A. Hendley, Essex, UK) by adding one drop to each well and immediately sucking off the liquid and used as antigens for indirect fluorescent antibody (IFA) test.

Test procedure: The IFA test was carried out based on the procedure described by Goddeeris *et al.* [10]. Multispot antigen slides prepared from the *T. parva* infected cells as described above were tested. The first two wells on each slide were served as negative and positive controls. *T. parva* Lusaka-infected lymphoblasts were served as positive control. MAbs were diluted in PBS to 1:200, and further two-fold serial dilutions were made. Rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate (Miles Laboratories, Slough) was diluted at a concentration of 1:1,000 in PBS containing 0.01% Evans blue. Finally, slides were examined for fluorescence using a fluorescence microscope.

The reaction was regarded as positive case when fluorescence was observed at dilutions greater than 1: 200 of MAbs. In this paper, when MAbs gave fluorescence at dilutions of 1: 200 to 1: 400, 1: 400 to 1: 800, or greater than 1: 800, the reactions were recorded as mild, medium, or strong positive reaction, respectively.

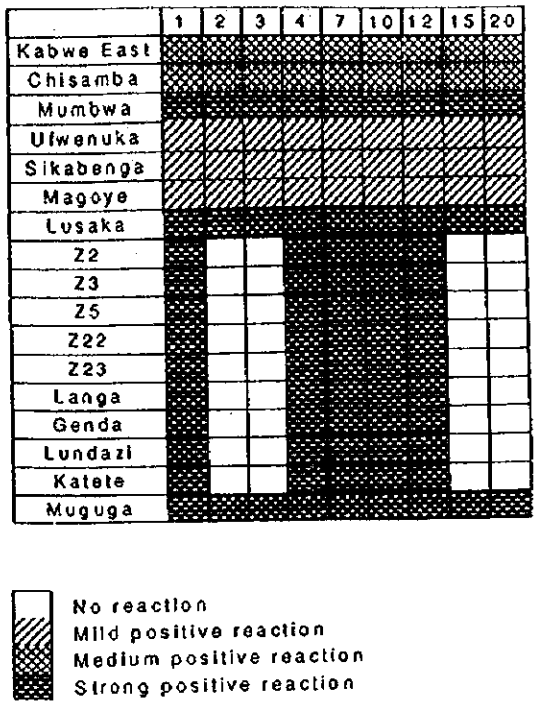

Fig. 2. Anti-schizont monoclonal antibody profiles of 16 Zambian *Theileria parva* and of the Muguga cocktail. Asteriks indicate new isolates.

RESULTS

The MAb profiles of the 7 newly isolated *Theileria* - infected cell lines plus those of the previously established cell lines are shown in Fig. 2. They also differed markedly with the MAb profiles of the old stocks from Eastern and Southern provinces which were previously characterized by ILRI (Fig. 2). The old stocks still gave negative reactions with MAbs 2, 3, 15, and 20. Although the MAb profiles for the newly isolated stocks from Southern, Central and Lusaka

Table 1. The origin of Zambian *Theileria parva* stocks used in this study

Isolate	History & year isolated	Province
Old:-		
Zambia 2	Established from Chiparamba, in 1982 by FAO	Eastern
Zambia 3	Established from Chitongu in 1982 by FAO	Southern
Zambia 5	Established from Mandali, Monze in 1983 by FAO	Southern
Zambia 22	Established from Mandali, Monze in 1985 by FAO	Southern
Zambia 23	Established from Kachomba, Choma in1985 by FAO	Southern
Katete	Established from Katete in 1985 by ASVEZA	Eastern
Langa	Established from Langa in1989 by ASVEZA	Eastern
Genda	Established from Genda in1985 by ASVEZA	Eastern
Lundazi	Established from Lundazi in 1985 by ASVEZA	Eastern
New:-		
Magoye	Established from Mazabuka south in 1994 by UNZA	Southern
Ufwenuka	Established from Magoye south in 1995 by UNZA	Southern
Sikabenga	Established from Magoye north in 1995 by UNZA	Southern
Lusaka	Established from Kakoma farm in 1995 by UNZA	Lusaka
Kabwe East	Established from Kabwe east in 1995 by UNZA	Central
Chisamba	Established from Chisamba in 1995 by UNZA	Central
Mumbwa	Established from Sibuyunji in 1995 by UNZA	Central

FAO, Food and Agricultural Organisation.
UNZA, University of Zambia.
ASVEZA , Belgium Assistance to the Veterinary Services of Zambia.

provinces were similar in that they all gave positive reactions with MAbs 2, 3, 15, and 20, only 60–70% of the schizont-infected cells reacted with MAbs 2, 3, 15, and 20. Even though the new stocks from Southern, Central and Lusaka provinces showed similar MAb profiles reacting with MAbs 2, 3, 15, and 20 in addition to those reacting with the old ones, the intensity of their reactions differed remarkably. The Mumbwa (Central province) and the Lusaka (Lusaka province) isolates showed strong reactions with almost all the MAbs they reacted with in general. The Kabwe East & Chisamba (Central province) isolates showed medium reactions while the Ufwenuka, Sikabenga and Magoye group showed mild positive reactions.

DISCUSSION

The results obtained from this study using *Theileria* anti-schizont MAbs reveal antigenic diversity among Zambian *Theileria* stocks. The results also revealed that the stocks newly isolated in the Southern province reacted differently from old stocks, and that *Theileria* stocks from the Central and Lusaka provinces exhibited similar reacting profiles as those of stocks from the Southern province.

The observation that certain Zambian isolates exhibited different reactions with MAbs (mild, medium, and strong reactions) and that specific MAbs reacted with only a portion of the schizont-infected cells indicate that these isolates may contain mixed populations which differ in their expression of antigenic determinants on schizonts. Similar reactions with anti-schizont MAbs were observed in some Zimbabwe *Theileria* isolates [13] and in some *Theileria* isolates from Kenyan buffalo which were found to consist of a number of distinctly different populations of *Theileria* parasites after cloning *in vitro* [4]. One of the major reasons for this change in character of the Southern stocks could be the characteristic carrier state of *Theileria* parasites in their mammalian hosts as described by Maritim *et al.* [14]. *T. parva* induces carrier state in cattle [8, 15]. Recovery from *T. parva* infection either naturally [2, 21] or following immunization [7] or treatment [8], may result in a persistent transmissible infection. Maritim *et al.* [14] also reported that after the infection and treatment method the MAb profiles of parasites from persistent infected animals appeared to be similar to the original immunizing stock. This finding is being confirmed by our results in that the isolates from the Southern and Central provinces are showing a binding profile similar to that of the original immunizing stock (Muguga cocktail). Since the cocktail was used in these provinces, the disease has spread rapidly and has even gone beyond its original borders into the Lusaka and Copperbelt provinces [16]. As already stated in this paper the disease in the Southern and Central provinces was originally diagnosed as CD, but one wonders the validity of this term. All the Zambian isolates were put in group B of *T. parva* together with *Theileria* stocks from other countries which did not react with MAbs 2, 3, 15 and 20 [11, 12]. In the advent of this change in character there may be need of testing the MAb profiles of *T. parva* parasites in the other countries especially in those countries where foreign stocks were used in the immunization programs. Dolan [9] stated that although the cocktail provides protection against most isolates its use in other

areas other than those from which the isolates were made carries with it the potential danger of introducing parasites of an antigenic type against which local cattle may have no immunity. For this reason the use of locally isolated stocks in the immunization programs is strongly advocated.

ACKNOWLEDGEMENTS. We wish to thank the University of Zambia (Graduate School) and the Japan International Cooperation Agency (JICA) for both the technical and financial support they rendered during this study. We also wish to thank the Belgian Animal Disease Control Project in Zambia (ASVEZA) and ILRI for the provision of some of the *Theileria* stocks and MAbs, respectively.

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CHARACTERISATION OF SOME *Theileria parva* FROM ZAMBIA USING MONOCLONAL ANTIBODIES

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A total of 16 bovine lymphocytic cell lines infected with *Theileria parva* schizonts were characterised using a panel of *Theileria* anti-schizont monoclonal antibodies (MAbs). Comparison of the *Theileria* stocks isolated, before (old) and after (new) the 'Muguga cocktail' from Kenya was used to immunise cattle in Zambia, revealed differences in their reactivity against MAbs. The new isolates show MAb profiles similar to that exhibited by the 'Muguga cocktail' which was used to vaccinate cattle against theileriosis in these areas between 1983 and 1989. These results suggest that the use of the 'Muguga cocktail' to vaccinate animals against theileriosis in Zambia may have introduced *Theileria* stocks with different antigenic properties.

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ORIGINAL PAPER

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Detection of *Trypanosoma congolense* and *T. brucei* subspecies in cattle in Zambia by polymerase chain reaction from blood collected on a filter paper

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Abstract To facilitate epidemiology studies of African trypanosomiasis in cattle in Zambia, we adapted a polymerase chain reaction (PCR) method using blood spotted on filter papers. For easy preparation of template DNA from the dried blood, we adapted a simple DNA extraction method using Chelex-100, an anion-exchange resin. Using primers directed for repetitive nuclear DNA sequences, species-specific DNA amplifications were detected from the blood of rats infected with Zambian isolates of *T. congolense* and *T. brucei* subspecies. The method was sensitive enough to detect a single trypanosome for both species. In the Eastern Province of Zambia, 240 cattle were examined for motile flagellates in the buffy coat by the microhematocrit method, and 100 of them were positive for the test. These 100 animals were further examined by thin blood smears and PCR for species identification. The thin blood smear revealed 62 and 14 animals with *T. congolense* and *T. brucei* subspecies infection, respectively, whereas the PCR detected 73 of the former and 38 of the latter species. These results indicate that dried blood spots on filter papers are a useful source of DNA for detection of African trypanosomes by PCR.

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Introduction

Trypanosomiasis represents a considerable economic problem in cattle in Zambia. Although three major trypanosome species, *Trypanosoma congolense*, *T. vivax*, and *T. brucei* subspecies, have been reported from cattle and tsetse flies in Zambia (Awan and Sawchuck 1976; Chitambo and Arakawa 1991; Woolhouse et al. 1994), their distribution is not clearly elucidated because of limitations in detection and identification techniques. Meanwhile, the most important mode of transmission is mainly cyclic through tsetse flies; to a lesser extent, mechanical transmission may also play a role where hemophagous insects exist. For the control of African trypanosomiasis in Zambia, extensive epidemiology studies are a prerequisite. Thus, we have conducted the detection of trypanosomes in blood smears by conventional microscopic parasitological examination and through detection of antibodies in sera by enzyme-linked immunosorbent assay (Luckins 1977), but the specificity and the sensitivity of these methods are quite limited for individual diagnosis.

A method of polymerase chain reaction (PCR) has been introduced into diagnostic tests for detection of infection with African trypanosomes in humans and animals as well as tsetse flies. Specific repetitive nuclear DNA sequences can be amplified for *T. vivax*, *T. simiae*, and each of the three *T. congolense* subgroups Savannah, Forest, and Kenya Coast (Moser et al. 1989; Artama et al. 1992; Masiga et al. 1992). A common primer set is also available for detection of three *T. brucei* subspecies: *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodesiense* (Moser et al. 1989).

In the preparation of template DNA for PCR, a simple method using Chelex-100 has been reported for the extraction of malarial DNA from a small amount of whole blood or blood spots blotted on filter papers (Wooden et al. 1992, 1993; Kain et al. 1993). Collection of blood on filter papers is a very useful technique for wide-scale field surveys. Furthermore, detection of an-

tibodies from the dried blood samples is a well-established method in serodiagnosis for a number of diseases, including African trypanosomiasis (Robson and Ashkar 1972). Since we had been using blood-spot samples blotted on filter papers for the detection of antibodies against trypanosomes or parasite circulation antigens, we adapted the simple Chelex-100 DNA isolation method from blood spots on filters for PCR. This was the first trial of PCR detection of trypanosomes in cattle in Zambia.

Materials and methods

Parasites

We used a *Trypanosoma brucei* subspecies isolate obtained from a cow at Chisamba in Zambia in 1988 and a *T. congolense* isolate acquired from a cow at Petauke in Zambia in 1995. These parasites were parasitologically identified by microscopic examination and have been maintained by inoculation into experimental rats or cryopreservation in liquid nitrogen.

Infection of rats with trypanosomes and preparation of infected blood specimens

Cryopreserved blood-stage tryponastigotes of *T. congolense* or *T. brucei* subspecies were thawed and inoculated into the rat peritoneum. Several days later, rat peripheral blood was collected in the presence of citrate buffer (50 m*M* sodium citrate and 50 m*M* glucose, pH 7.2) and the parasites were counted by a hemocytometer. One part of the infected blood was directly frozen at −20 °C. Another part was serially diluted with citrate buffer, and 10 µl of diluents were blotted on filter papers (Whatman Number 41). The filters were dried and stored at −20 °C or kept at room temperature in a desiccated condition before use.

Blood collection from cattle and blood examination by the microhematocrit and thin-blood-smear method

Animals used in this study were locally bred, mostly of the indigenous Angoni breed of cattle, in ten crush pens in Petauke district in the Eastern Province of Zambia. Blood was collected into capillary tubes from the ear vein of 240 randomly selected cattle. The tube was centrifuged for 5 min at 15,000 rpm to obtain the buffy coat. A drop of the buffy coat was placed on a microscopic glass slide and examined for motile parasites under a light microscope at 400× magnification. Trypanosome search was done in up to 100 microscopic fields. A total of 100 cattle testing positive for motile flagellates in the buffy coat were further examined by thin blood smears. Blood was collected into capillary tubes again and a drop of whole blood was placed on a microscopic glass slide to make a thin blood smear. The smears were air-dried, fixed with methanol, and stained with 10% Giemsa solution (pH 7.2). The thin smears were examined under the oil-immersion objective lens. Another drop of whole blood was placed on Whatman 41 filter paper to make a blood spot for PCR. The blood spots were air-dried and stored at −20 °C until use.

Isolation of trypanosome DNA by Chelex-100 from rat and cattle blood specimens

DNA isolation by Chelex-100 was performed with a modification of the methods described by Walsh et al. (1991) and Wooden et al. (1993). A 10-µl aliquot of the frozen rat blood was added to 1 ml of 0.15% saponin (Sigma, USA) in phosphate-buffered saline (PBS, pH 7.2) in a 1.5-ml microcentrifuge tube for removal of red blood cells for 5–10 min on ice. Parasites were collected by centrifugation at 10,000 rpm for 1 min and washed once with cold PBS. The resultant cell pellet was added to 200 µl of 5% Chelex-100 (Bio-Rad, USA) in distilled water, incubated at 56 °C for 15 min, and subjected to boiling water for 8 min. The Chelex was removed by centrifugation at 10,000 rpm for 1 min, and the resultant supernatant was saved in a fresh tube. A 2-µl aliquot of the supernatant was used for PCR amplification. For dried blood samples from rats and cattle, a small piece (about 5 mm^2) of filter paper with a 10-µl blood spot was initially soaked in 0.15% saponin in PBS and processed as described above.

PCR amplifications

Standard PCR amplifications were carried out in 10-µl reaction mixtures containing 10 m*M* TRIS-HCl (pH 9.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 125 µ*M* each of the four deoxynucleoside triphosphates, primers at 0.25 m*M*, 2 µl of Chelex-isolated DNA solution, and 0.5 units of Taq DNA polymerase (Pharmacia, USA). The reaction mixtures were overlaid with 20 µl paraffin oil and cycled in a programmable heating block (Astek Inc. Japan) as follows: samples were incubated at 94 °C for 3 min in an initial denaturing step and were subjected to 40 cycles involving denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The 10-µl PCR products were electrophoresed through 2% agarose in 1 × TAE [40 m*M* TRIS-acetate and 1 m*M* ethylenediaminetetraacetic acid (EDTA)] at 100 V. Gels were stained with a 0.5 µg/ml concentration of ethidium bromide and photographed.

Primer sets for repetitive nuclear DNA sequences of different trypanosome species, subspecies, and subgroups were referred to as reported sequences (Kirchhoff and Donelson 1993) and synthesized as follows: TBR1 (CGAATGAATATTAAACAATGCGCAG) and TBR2 (AGAACCATTTATTAGCTTTGTTGC), for *T. brucei* subspecies; TCN1 (TCGAGCGAGAACGGGCACTTTGCGA) and TCN2 (ATTAGGGACAAACAAATCCCGCACA), for the *T. congolense* Savannah subgroup; TCF1 (GGACACGCCAGAAGGTACTT) and TCF2 (GTTCTCGCACCAAATCCAAC), for the *T. congolense* Forest subgroup; TCK1 (GTGCCCAAATTTGAAGTGAT) and TCK2 (ACTCAAAATCGTGCACCTCG), for the *T. congolense* Kenya Coast subgroup; TVW1 (CTGAGTGCTCCATGTGCCAC) and TVW2 (CCACCAGAACACCAACCTGA), for *T. vivax*; and TSM1 (CCGGTCAAAAACGCATT) and TSM2 (AGTCGCCCGGAGTCGAT), for *T. simiae*.

Results

Specificity of PCR detection of *Trypanosoma congolense* and *T. brucei* subspecies from wet and dried blood of infected rats

A 10-µl aliquot of frozen whole blood containing 5×10^5 trypanosomes of *T. congolense* or a 10-µl blood spot on a filter was subjected to DNA extraction, and resultant DNA solutions were saved in a tube in a volume of 200 µl. In all, 2 µl of the DNA suspension, an amount of template DNA equivalent to a DNA content of 5×10^3 parasites, was used for PCR. When a primer set of TCN (TCN1 and TCN2) was used, a large amount of amplified DNA was observed in a gel from both wet and dried blood materials (Fig. 1A). The size of the PCR product was near 326 bp, which were supposed to be amplified with the TCN primer set from 369-bp repeats of the *T. congolense* Savannah subgroup (Masiga et al.

1992). Thus, the *T. congolense* isolate in Zambia appears to belong to the Savannah subgroup and not to the Forest subgroup, which is detectable by the TCF primer set (TCF1 and TCF2), or to the Kenya Coast subgroup, which is detectable by TCK primers (TCK1 and TCK2). A non-specific band was observed with a primer set of TVW (TVW1 and TVW2) in the whole blood material but not in the dried blood material on the filter paper (Fig. 1A).

Likewise, rats were infected with trypomastigotes of a Zambian isolate of *T. brucei* subspecies. At 6 days after the infection, although it was hard to detect motile parasites in the fresh, wet blood-film preparation under a microscope, we confirmed a trypanosome in a Giemsa-stained blood smear. DNA extraction was then performed from 10 μl whole blood or 10 μl dried blood spots on a filter paper as described above. When a primer set of TBR (TBR1 and TBR2) was used, a large amount of PCR product was detected from both wet and dried blood materials (Fig. 1B). The size of the PCR product was near 173 bp, which were amplifiable from 177-bp repeats of *T. brucei* subspecies with the TCN primer set (Masiga et al. 1992). Some nonspecific DNA bands were observed with TVW and TCN primers in the whole blood sample but not in the dried blood sample (Fig. 1B).

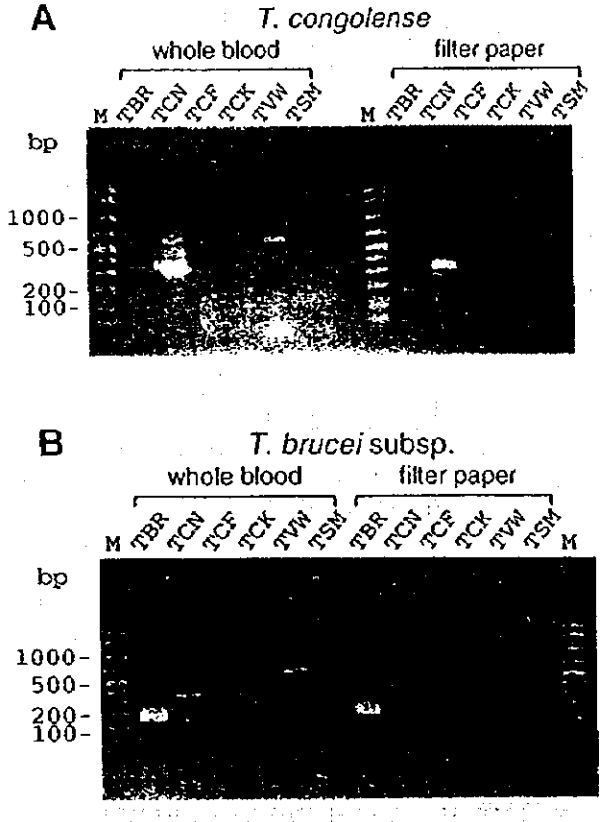

Fig. 1 A,B PCR amplification of repetitive nuclear DNA sequences of A *Trypanosoma congolense* and B *T. brucei* subspecies. DNA extracted by Chelex-100 from infected rat whole blood or blood spotted on filter papers was subjected to PCR with different primer sets. (*M* DNA molecular standards, *TBR* TBR1 and TBR2, *TCN* TCN1 and TCN2, *TCF* TCF1 and TCF2, *TCK* TCK1 and TCK2, *TVW* TVW1 and TVW2, *TSM* TSM1 and TSM2)

Sensitivity and stability of PCR detection of *T. congolense* and *T. brucei* subspecies repetitive nuclear DNA sequences

For examination of the sensitivity of the PCR detection technique adopted, a series of 10-fold dilutions of DNA extract from *T. congolense*-infected whole blood were examined. The diluent contained an amount of DNA equivalent to 5×10^3 to 5×10^{-5} trypanosomes. Specific DNA amplifications were observed in the presence of DNAs up to decreased amounts equivalent to 5×10^{-4} parasites (Fig. 2). This suggests that at least 2,000 copies of target repetitive sequences were produced in a single trypanosome. Moser et al. (1989) reported that the 369-bp repeats of *T. congolense* constituted about 5% of the nuclear DNA. This implies that the repetitive sequences are present at about 5,400 copies in a single parasite if the genome size is 4×10^7 bp.

In a different experiment, we examined the sensitivity of PCR detection of *T. brucei* subspecies. Infected fresh rat blood (7.7×10^7 trypanosomes/ml) was diluted and blood spots were made on filter papers such that each 10-μl sample of diluent contained from a single parasite to 1×10^4 trypanosomes. DNA was extracted by Chelex-100 from each blood spot and subjected to PCR with TBR1 and TBR2 primers. Specific DNA amplification was detected even from the blood spot that contained a single parasite (data not shown). In terms of the sensitivity of PCR using the TCN and TBR primers, Moser et al. (1989) reported that a visible amplification band was present for both *T. congolense* and *T. brucei* subspecies when the reaction was performed on 0.1 pg

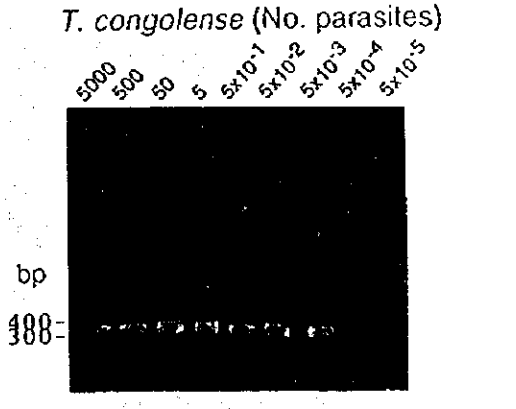

Fig. 2 Sensitivity of PCR detection of *T. congolense*. The PCR reaction mixture contained the TCN primer set and an amount of DNA equivalent to 5×10^3 to 5×10^{-5} trypanosomes

parasite DNA, which is about the same amount of DNA calculated to be present in a single trypanosome.

To examine the stability of trypanosome DNA on filter papers, we compared PCR detection of *T. congolense* from blood spots on filters stored at −20 °C and those that were stored at room temperature. After storage of each blood sample for 3 months, DNAs were extracted, diluted, and subjected to PCR. Specific DNA amplification was detected from dried blood stored at −20 °C without decreased sensitivity, but from those that had been stored at room temperature, a 1,000-fold decrease in sensitivity was observed (data not shown).

Detection of *T. congolense* and *T. brucei* subspecies from cattle blood by thin blood smears and PCR

We examined thin blood smears of the 100 cattle in which we had detected motile flagellates in the buffy coat by the microhematocrit method. In the smears, we determined 62 individuals of *T. congolense*, 23 of *T. vivax*, and 14 of *T. brucei* subspecies on the basis of their morphological features. Only in one smear did we fail to detect any trypanosome. Mixed infections were found as follows: nine for *T. congolense* and *T. vivax*, two for *T. congolense* and *T. brucei* subspecies, three for *T. vivax* and *T. brucei* subspecies, and three for a combination of all the three species.

The PCR was performed with the dried blood samples on filter papers collected from these 100 animals. When primers sets of TCN and TBR were used, species-specific PCR amplifications were detected. A typical result of PCR amplifications is shown in Fig. 3. The PCR revealed 73 *T. congolense* and 38 *T. brucei* subspecies infections, including 29 mixed infections. These results indicated that the PCR method was much more sensitive than the thin-blood-smear method in detecting trypanosomes, especially *T. brucei* subspecies, in naturally infected cattle (Table 1).

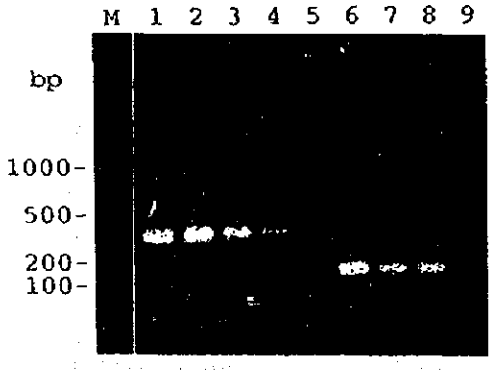

Fig. 3 PCR amplification of repetitive nuclear DNA sequences of *T. congolense* and *T. brucei* subspecies from cattle blood spotted on filter papers. Nine individual cattle were the sources of blood for PCR with TCN1 and TCN2 primers (*lanes 1–5*) or TBR1 and TBR2 primers (*lanes 6–9*)

Table 1 Comparison of the thin-blood-smear method and the PCR for detection of *Trypanosoma congolense* and *T. brucei* subspecies in cattle

PCR	Thin blood smear (number of animals)		
	Positive	Negative	Total
T. congolense:			
Positive	50	23	73
Negative	12	15	27
Total	62	38	100
T. brucei subspecies:			
Positive	5	33	38
Negative	9	53	62
Total	14	86	100

Discussion

In this study, we showed that a small amount of cattle blood on filter papers was a useful DNA source for PCR detection of two *Trypanosoma species*, *T. congolense* and *T. brucei* subspecies. This PCR method was sensitive enough to detect a single trypanosome.

Diagnosis of trypanosomes in animals in Zambia has been done using the conventional parasitological methods often confirmed by examination of Giemsa-stained blood smears. Results obtained by this method have shown a higher prevalence of *T. congolense* infections than of infestation with *T. vivax* and *T. brucei* subspecies (Awan and Sawchuck 1976). The results obtained in thin blood smears from 100 cattle in this study also showed a higher prevalence of *T. congolense* infections, followed by infestation with *T. vivax* and *T. brucei* subspecies, with the species infection ratio being 62:23:14 (4.4:1.6:1), yet only two mixed infections for *T. congolense* and *T. brucei* subspecies were seen. However, the PCR technique adopted in this study showed that the rate of *T. congolense* and *T. brucei* subspecies infections was 72 to 38 (1.9:1). The PCR revealed much a higher prevalence of *T. brucei* subspecies infections and detected 29 mixed infections for these 2 species. On the other hand, we have seen circulating antigens of *T. brucei* subspecies in 58 of 100 sera collected from these cattle by enzyme-linked immunosorbent assay (Lubinga 1995). These results therefore indicate that the PCR technique is an another sensitive diagnostic tool for not only trypanosome identification but also detection of mixed trypanosome infections. With regard to *T. vivax*, we saw PCR amplifications in some cattle blood samples using the TVW primer set. However, further studies are needed to confirm our preliminary results.

For most African trypanosome species, repetitive nuclear DNA sequences have been used as targets for PCR amplification since the minicircle kinetoplast DNA sequences are highly heterogeneous (Kirchhoff and Donelson 1993). However, the satellite repeats are also variable among isolates of *T. congolense* in Africa. An alignment of the nucleotide sequences showed only 47–71% identity among the Savannah, Forest, and Kenya

Coast subgroups of *T. congolense*, although the size of the repeats is about 370 bp in all three groups (Masiga et al. 1992). The present study also revealed that a *T. congolense* isolate and a number of trypanosomes infecting cattle in Petauke in the Eastern Province of Zambia appeared to have repetitive nuclear DNAs similar to that found in the Savannah subgroup in Kenya. Interestingly, 12 cattle were found to be positive for *T. congolense* infection by the thin-blood-smear method but proved to be negative as tested by the PCR method with TCN primers (Table 1). These samples were also negative as determined by PCR with the TCF and TCK primer sets for other *T. congolense* subgroups. The reasons for this discrepancy may be that variations exist in the repetitive nuclear DNA sequences of some Zambian strains or that the level of parasitemia was very low. A similar situation may also apply to the *T. brucei* subspecies. Further isolation and characterization of trypanosomes in Zambia will answer these questions.

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