5 RESULTS

5.1 Propagation of *T. congolense* stocks and clones

The study stocks from Ethiopia and Burkina Faso and the clones derived from them were successfully propagated in *M. coucha*. The pre-patent period for the field isolates from Ethiopia (PA 073, PA 077), and clones derived from them, ranged from 6 to 13 days. The stocks from Burkina Faso (SA 267, SA 268 and SA 95) and clones derived from them had pre-patent periods ranging from 6 to 10 days. The trypanosomes were sub-passaged 1-2 times in *M. coucha* before initiating cloning and performing the Standard Mouse Test.

5.2 Cloning of *T. congolense*

Twelve to fifteen clones were obtained from each stock of *T. congolense* isolated from cattle in Ethiopia and Burkina Faso. Each clone was inoculated into a mouse, which was immunosuppressed with 300 mg/kg bw cyclophosphamide (Endoxan®) 24 hours prior to infection. Two to three clones were successfully derived in mice from each of the stocks with cloning success rates ranging from 16.67 to 25%. The overall cloning success rate was 20.3% (Table 9).

Table 9: Percent success rates of cloning of *T. congolense* stocks isolated from cattle in Ethiopia and Burkina Faso

Trypanosome stocks	No. of attempts	Clones generated	% Success rate
MBOI/ET/97/PA 073	15	3	20
MBOI/ET/97/PA 077	15	3	20
MBOI/BK/98/SA 095 2308	15	3	20
MBOI/BK/89/SA 0267	12	3	25
MBOI/BK/89/SA 0268	12	2	16.67
Total	69	14	20.3

5.3 Isometamidium sensitivity of *T. congolense* clones

5.3.1 *T. congolense* reference clones

T. congolense reference clones (IL 2642, IL 1180) with known isometamidium sensitivity phenotype were used as sensitive controls. They were tested in parallel with the study clones using the standard mouse test (Eisler et al., 2001). The results showed that the two reference clones were highly sensitive to isometamidium as low as 0.01 mg/kg bw in mice. All the mice infected with IL 2642 were cured when treated with isometamidium at dosages of 0.1 to 20 mg/kg bw. Five out of 6 mice were cured when they were treated with 0.01 mg/ kg bw isometamidium. The isometamidium CD_{50} value of this clone was estimated at 0.0086 mg/kg bw (95% CI: - 0.0047 – 0.022). With regard to IL 1180, no parasitaemia was detected for 60 days post treatment with isometamidium at dosages of 0.5 to 20 mg/kg bw. Five of the six mice infected were cured when treated with 0.01 and 0.1 mg/kg bw isometamidium, respectively. The isometamidium CD_{50} value of this clone of parasite was estimated at 0.022 mg/kg bw (95% CI: - 0.14 – 0.18). There was no significant ($p > 0.05$) difference in the isometamidium CD_{50} values of the two reference clones. Thus, these clones were characterised as highly sensitive to isometamidium. Analyses of the means of relapse intervals and CD_{50} values for isometamidium of these reference clones (IL 2642 and IL 1180) are given in Table 10.

Table 10: Summary results of the isometamidium sensitivity analysis of *T. congolense* reference clones in mice

Clones	Drug dosage (mg/kg bw)	No. relapsed/ treated	Mean relapse interval in days	SD	Min.	Max.
IL 2642	0.0	6/6	7.17[a]	1.17	6	9
	0.01	1/6	55.33[b]	13.88	27	61+
	0.1	0/6	61.00[b]	0.00	61+	-
	0.5	0/6	61.00[b]	0.00	61+	-
	3.0	0/6	61.00[b]	0.00	61	-
	20.0	0/6	61.00[b]	0.00	61+	-
IL 1180	0.0	6/6	15.17[a]	7.6	9	28
	0.01	1/6	55.50[b]	13.47	28	61+
	0.1	1/6	55.83[b]	12.66	30	61+
	0.5	0/6	61.00[b]	0.00	61+	-
	3.0	0/6	61.00[b]	0.00	61+	-
	20.0	0/6	61.00[b]	0.00	61+	-

[a,b] Within clone, means with different superscripts differed significantly (p=0.001)
SD= standard deviation; + mice with no relapse infection for 60 days after treatment and considered cured (for statistical purposes, animals which did not relapse after treatment were assigned a value of 61), Min.= Minimum, Max.= Maximum.

5.3.2 *T. congolense* clones from Ethiopia

Four clones were derived from stocks of *T. congolense*, three from PA 77 and one from PA 73. All the clones were derived from the stocks without drug selection and were characterised for their sensitivity to isomatemidium chloride in mice using SMT (Eisler et al., 2001). The results showed that all the four clones expressed high levels of resistance to isometamidium. Results of analyses of the means of relapse intervals after treatment with different doses of isometamidium for each of the clones from Ethiopia are shown in Table 11. None of the mice infected with clones PA 77 clone 1 and PA 077 clone 2 were cured when treated with isometamidium at dosages of 0.01 to 0.5 mg/kg bw. Only 2/6 (33.33%) and 4/6 (66.67%) of mice infected with PA 77 clone 1 were cured by 3.0 mg/kg bw and by 20 mg/kg bw of isometamidium, respectively. With regard to PA 077 clone 2, 4/6 (66.67%) and 5/6 (83.3%) of mice infected were cured by 3.0 mg/kg and by 20.0 mg/kg bw isometamidium, respectively. None of the mice infected with PA 073 clone 1 was cured when treated with isometamidium at dosages of 0.01 to 3 mg/kg bw. However, all of the six mice infected with the same clone were cured when 20 mg/kg bw isometamidium was administered. Within each

of the clones, mice treated with lower doses showed relapse infections earlier (p = 0.001) than mice treated with higher doses (Table 11).

Table 11: Summary results of the isometamidium sensitivity of *T. congolense* clones derived from parental stock (PA 077 and PA 73) from Ethiopia in mice

Clones	Drug dosage (mg/kg bw)	No. relapsed/ treated	Mean of relapse interval in days	SD	Min.	Max.
PA 77 Clone 1	0.0	6/6	11.67[a]	5.16	6	19
	0.01	6/6	11.33[a]	2.07	10	14
	0.1	6/6	8.00[a]	1.26	6	12
	0.5	6/6	13.17[a]	2.71	10	17
	3.0	4/6	30.83[ab]	23.74	10	61+
	20.0	2/6	43.67[b]	26.86	8	61+
PA 77 Clone 2	0.0	6/6	8.83[a]	2.04	8	13
	0.01	6/6	9.00[a]	1.55	8	11
	0.1	6/6	12.67[a]	3.50	8	18
	0.5	6/6	13.50[a]	3.94	8	18
	3.0	2/6	46.17[b]	23.00	15	61+
	20.0	1/6	53.83[b]	17.55	18	61+
PA 77 Clone 3	0.0	6/6	9.83[a]	2.04	9	14
	0.01	6/6	10.83[a]	2.99	9	16
	0.1	6/6	9.55[a]	1.22	9	12
	0.5	6/6	10.33[a]	2.16	9	14
	3.0	4/6	37.83[b]	25.58	9	61+
	20.0	0/6	61.00[c]	0.00	61+	-
PA 73 Clone 1	0.0	6/6	8.83[a]	0.41	8	9
	0.01	6/6	9.00[a]	0.63	8	10
	0.1	6/6	8.50[a]	0.55	8	9
	0.5	6/6	10.00[a]	2.00	9	14
	3.0	6/6	17.67[b]	5.16	9	23
	20.0	0/6	61.00[c]	0.00	61+	-

[a,b,c] Within clone, means with different superscripts differed significantly (p=0.001)
SD= standard deviation; + mice with no relapse infection for within 60 days after treatment were considered cured (for statistical purposes, animals which did not relapse after treatment were assigned a value of 61); Min., Minimum; Max., Maximum

Results of determinations of the isometamidium CD_{50}´s for the clones are given in Table 12. The isometamidium CD_{50} values for the clones from Ethiopia ranged between 8.88 and 13.37 mg/kg bw. By contrast, the values for the drug-sensitive clones, IL 2642 and IL 1180, were 0.0086 mg/kg and 0.022 mg/kg bw, respectively. There was no significant (p>0.05) difference in expression of resistance to isometamidium among the 3 clones derived from the same

parental stock (PA 077). The PA 73 clone 1 derived from parental stock PA 73 gave an estimated CD_{50} value of 13.37 mg/kg bw, which was not significantly ($p>0.05$) different from the rest of the clones from Ethiopia. Therefore, compared to the sensitive reference clones (IL 2642 and IL 1180), all four clones from Ethiopia showed significantly ($p<0.05$) high levels of resistant to isometamidium. They were 480 to 608-fold more resistant to isometamidium than the sensitive reference clones.

Table 12: Results of determinations of CD_{50} values of isometamidium in mice for *T. congolense* study clones

Clone/Stock	**CD_{50} (mg/kg bw)§**	**95% Confidence interval**
IL 2642‡	0.0086[a]	- 0.0047 - 0.022
IL 1180‡	0.022[a]	- 0.137 - 0.182
PA 77 Clone 1	12.81[b]	8.39 - 17.23
PA 77 Clone 2	8.99[b]	5.23 - 12.77
PA 77 Clone 3	9.86[b]	5.56 - 14.16
PA 73 Clone 1	13.37[b]	7.99 - 18.76
SA 095 2308 Clone 2	20.00[c]	18.61 - 21.39
SA 095 2308 Clone 3	19.80[c]	19.19 - 20.41
SA 267 Parental stock	> 20.00[†]	-
SA 267 Clone 1	> 20.00[†]	-
SA 267 Clone 2	> 20.00[†]	-
SA 267 Clone 3	> 20.00[†]	-
SA 268 Clone 1	> 20.00[†]	-

‡ Reference sensitive clone
§ Dosages of isometamidium required to cure approximately 50% of treated mice
[a,b,c] CD_{50} values with different superscripts differed significantly ($p<0.05$)
[†] CD_{50} value of isometamidium greater than 20 mg/kg bw

5.3.3 *T. congolense* clones from Burkina Faso

Table 13 shows the results of the isometamidium sensitivity analysis performed using the standardised drug sensitivity test in mice for the *T. congolense* clones from Burkina Faso. Three clones were derived from the parental stock SA 095 without drug selection and designated as SA 095 clone 1, 2 and 3. Two of the clones, SA 095 clone 2 and SA 095 clone

3, were tested for isometamidium sensitivity in mice. All the mice infected with these clones were parasitaemic within 8 to 18 days of treatment with isometamidium at dosages of 0.01 to 3.0 mg/kg bw. Only 3/6 (50%) of the mice infected with SA 095 clone 2 and 4/6 (66.67%) of the mice infected with SA 095 clone 3 were cured by 20 mg/kg bw isometamidium. For each of the clones, there were trends among the dosages of isometamidium administered and the means of relapse intervals in days. The mean relapse intervals for the groups that received the highest dosages were significantly ($p=0.001$) higher than those which received lower dosages (Table 12). Mice treated with the lowest drug dosages showed relapse of infections earlier than those treated with highest dosages.

The isometamidium CD_{50} values of these two clones were 20.0 (95% CI: 18.61 – 21.39) and 19.8 (95% CI: 19.19 – 20.41), respectively. There was no significant variation between the isometamidium CD_{50} values of these two clones. Neverthless, these values were significantly higher than those obtained for the clones from Ethiopia (Table 12). When compared to the two isometamidium-sensitive clones, IL 2642 and IL 1180, both clones from Burkina Faso were at least 900-fold more resistant to isometamidium.

Similarly, determinations of sensitivity to isometamidium of *T. congolense* SA 267 stock and three clones derived from it were performed using SMT. The results (Table 13) showed that the parental stock and its derived clones exhibited high levels of resistance to isometamidium such that none of the test mice was cured when treated with isometamidium at dosages of 0.01 to 20 mg/kg bw. Mice treated with 20 mg/kg bw isometamidium had mean relapse intervals significantly ($p=0.001$) higher than those treated with lower dosages.

The clone derived from *T. congolense* stock SA 268 from Burkinal Faso also gave a high level of resistance to isometamidium with the test-mice having relapses within 8 to 28 days of treatment. For this clone, there existed no significant ($p>0.05$) variations in the means of relapse intervals among the different dosage-treatment groups. The isometamidium CD_{50} values of the parent SA 267 and its derived clones as well as the clone derived from SA 268 could not be established, as none of the mice treated was cured with the ranges of drug dosages used (Table 13). When compared to the IL 2642 and IL 1180 clones, these clones of parasites expressed at least 1000-fold resistance to isometamidium.

Figure 2 shows the comparison of the probability of 'survival curves' of stocks and clones from Ethiopia and Burkina Faso in relation to the reference clones IL 2642 and IL 1180, in response to treatment with isometamidium. For any concentration of isometamidium the rate of decline in 'survivability' (elimination of parasitaemia) was slower in the clones from Burkina Faso than the ones from Ethiopia. Thus, all the study clones from Ethiopia expressed significantly ($p<0.05$) lower levels of resistance to isometamidium chloride than the clones from Burkina Faso. Both the isometamidium sensitive reference clones IL 2642 and IL 1180 showed the fastest decline in 'survivability'. The study clones derived from SA 095 stock of Burkina Faso (SA 095 clone 1 and 2) appeared to express significantly lower ($p<0.05$) levels of resistance to isometamidium than the clones derived from SA 0267 and SA 0268.

Table 13: Summary results of the isometamidium sensitivity of *T. congolense* stock and clones from Burkina Faso in mice

Trypanosome	Drug dosage (mg/kg bw)	No. relapsed/ treated	Means of relapse intervals in days	SD	Min.	Max.
SA 095 Clone 2	0.0	6/6	7.00[a]	0.00	7	7
	0.01	6/6	7.33[a]	0.82	7	9
	0.1	6/6	7.00[a]	0.00	7	7
	0.5	6/6	8.67[a]	4.08	7	17
	3.0	6/6	9.67[a]	0.52	9	10
	20.0	3/6	34.00[b]	29.58	7	61+
SA 095 Clone 3	0.0	6/6	6.83[a]	0.75	6	8
	0.01	6/6	7.00[a]	0.63	6	8
	0.1	6/6	7.50[a]	1.23	7	10
	0.5	6/6	6.00[a]	3.74	7	10
	3.0	6/6	11.67[a]	2.16	8	13
	20.0	2/6	46.50[b]	23.25	8	61+
SA 267 Parent	0.0	6/6	8.83[a]	0.41	8	9
	0.01	6/6	8.67[a]	2.58	7	12
	0.1	6/6	12.33[a]	0.82	12	14
	0.5	6/6	12.00[a]	0.00	12	12
	3.0	6/6	13.00[a]	1.67	12	16
	20.0	6/6	19.40[b]	8.47	12	33
SA 267 Clone 1	0.0	6/6	8.00[a]	1.67	7	11
	0.01	6/6	10.50[a]	3.67	9	18
	0.1	6/6	8.67[a]	1.97	7	11
	0.5	6/6	7.33[a]	0.82	7	9
	3.0	6/6	7.33[a]	0.82	7	9
	20.0	6/6	15.00[b]	4.10	9	21
SA 267 Clone 2	0.0	6/6	6.33[a]	1.63	5	9
	0.01	6/6	6.33[a]	1.03	5	7
	0.1	6/6	6.50[a]	1.98	5	10
	0.5	6/6	5.67[a]	1.03	5	7
	3.0	6/6	8.17[a]	1.33	7	10
	20.0	6/6	14.33[b]	0.82	14	16
SA 267 Clone 3	0.0	6/6	7.33[a]	0.82	7	9
	0.01	6/6	8.17[a]	2.99	6	12
	0.1	6/6	7.83[ac]	2.56	6	13
	0.5	6/6	9.50[ac]	1.23	6	13
	3.0	6/6	11.83[bc]	1.60	9	12
	20.0	6/6	13.67[b]	3.68	9	20
SA 268 Clone 1	0.0	6/6	9.17[a]	7.37	7	16
	0.01	6/6	12.33[a]	7.82	8	28
	0.1	6/6	11.33[a]	3.93	8	18
	0.5	6/6	9.83[a]	2.40	8	14
	3.0	6/6	8.00[a]	0.00	8	8
	20.0	6/6	21.33[a]	19.94	8	21

[a,b,c] means within each clones/stock followed by different superscripts differ significantly (p=0.001)
SD, standard deviation; +, mice with no relapse infection for within 60 days after treatment were considered cured (for statistical purposes, animals which did not relapse after treatment were assigned a value of 61); Min., Minimum; Max., Maximum

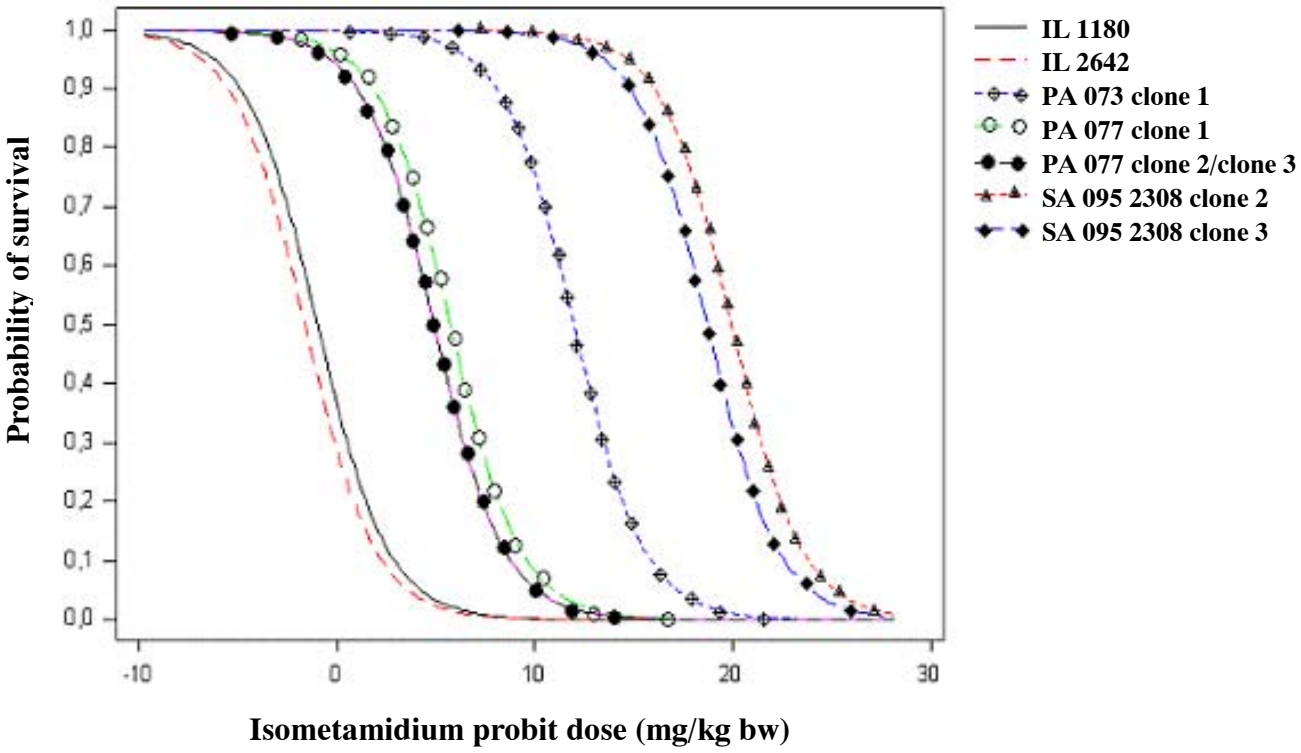

Figure 2: Parametric survival plot of infections with clones of *T. congolense* in response to treatment with different doses of isometamidium in mice. The predicted probabilities at which infections were cured were calculated as 1-probability of survival of trypanosomes.

5.4 Experimental induction of isometamidium-resistance in *T. congolense* using immunosuppressed mice

The *T. congolense* reference clone IL 2642 that is highly sensitive to isometamidium (CD_{50} value of 0.007 mg/kg bw) (Wilkes et al., 1997) was used to generate isometamidium resistant populations. Mice (*M. coucha*) immunosuppressed with 300 mg/kg bw of cyclophosphamide (Endoxan®) were infected with 10^5 trypanosomes after 24 hours of immunosuppression. Following the infection they were repeatedly treated with sub-curative doses of isometamidium. The development of resistance of the trypanosome population in mice to this drug progressively increased over a period of 16 months. IL 2642 was passaged 8, 10 and 13 times to produce populations which showed relapse of infections to 0.3, 0.5, and 1 mg/kg bw of isometamidium, respectively (Figure 1). These populations were approximately 42, 71 and 143 times more resistant to the parental stock, respectively.

A high degree of resistance, comparable to the resistance level demonstrated in the field, was achieved in the current study. Initially only slight increases of dosage levels were possible. Once the organisms gained marked resistance, it was possible to increase the dosages significantly. Eight sub-passages over a period of about 11 months were required to attain relapses to 0.3 mg/kg bw. An additional 2 sub-passages, augmented by 3 more sub-passages, were sufficient to obtain trypanosomes that showed relapse of infections after treatment with 0.5 mg/kg bw and 1.0 mg/kg bw isometamidium, respectively. Schematic representation of the procedure followed for the induction of resistance in immunosuppressed mice and results are shown in Figure 1.

In order to evaluate the susceptibility of the *T. congolense* IL 2642 clone and its isometamidium-resistant derivatives to isometamidium chloride and diminazene aceturate in normal immunocompetent mice, the methodology of Eisler et al. (2001) was followed. Standard logit analyses were used to express the drug susceptibility of each population to each of the two trypanocides as a CD_{50}. The drug sensitivity studies in normal immunocompetent mice showed that the resistance was indeed genuine and stable (Table 14).

Table 14: Results of determination of sensitivity of *T. congolense* IL 2642 and its isometamidium-resistant derivative IL 2642 1R to isometamidium and diminazene in immunocompetent mice.

Clones	Drug dosage (mg/kg bw)	No. relapsed/ treated	Mean relapse interval in days	SD	Min.	Max.
IL 2642	Isometamidium					
	0.0	6/6	7.17[a]	1.17	6	9
	0.01	1/6	55.33[b]	13.88	27	61+
	0.1	0/6	61.00[b]	0.00	61+	-
	0.5	0/6	61.00[b]	0.00	61+	-
	3.0	0/6	61.00[b]	0.00	61	-
	20.0	0/6	61.00[b]	0.00	61+	-
IL 2642 1R	Isometamidium					
	0.0	6/6	7.33[a]	3.27	6	14
	0.1	6/6	6.33[a]	0.82	6	8
	0.5	5/6	20.83[ac]	19.82	8	14
	1.0	3/6	44.00[bc]	25.44	17	61+
	2.0	2/6	48.50[bc]	22.30	6	61+
	3.0	0/6	61.00[b]	0.00	61+	-
IL 2642	Diminazene					
	0.0	6/6	7.00[a]	0.00	7	7
	3.0	6/6	12.50[a]	4.72	11	21
	10.0	0/6	61.00[b]	0.00	61+	-
	20.0	1/6	55.83[b]	12.66	30	61+
	40.0	0/6	61.00[b]	0.00	61+	-
	60.0	0/6	61.00[b]	0.00	61+	-
IL 2642 1R	Diminazene					
	0.0	6/6	7.00[a]	1.10	6	8
	3.0	4/6	25.67[ab]	27.54	6	61+
	10.0	3/6	34.33[ab]	29.27	6	61+
	20.0	2/6	43.67[ab]	26.92	6	61+
	40.0	1/6	53.50[b]	18.37	16	61+
	60.0	1/6	52.83[b]	20.00	12	61+

[a,b,c] within clones, means with different superscripts differed significantly (p=0.001)
SD= standard deviation; + mice with no relapse infection for 60 days after treatment were considered cured (for statistical purposes, animals which did not relapse after treatment were assigned a value of 61); Min., Minimum; Max., Maximum

Over the 16-month period of drug selection, the resistance of *T. congolense* IL 2642 to isometamidium chloride was increased roughly 159-fold (from a CD_{50} of 0.0086 mg/kg bw to a CD_{50} of 1.37) (Table 15). The survival probabilities that infected mice remained infected after treatment with 1 mg/kg bw isometamidium were analysed. The results showed that only 1.5% (95% CI: 0.00 – 0.076) of the mice infected with the parental clone (IL 2642) survived

treatment (showed relapse of infection) with 1 mg/kg bw isometamidium. In contrast, about 68.43% (95% CI: 0.44 – 0.89) of the mice infected with the isometamidium-resistant derivative (IL 2642 1R) survived treatment (showed relapse of infection) when treated with 1 mg/kg bw isometamidium (Table 15).

When *T. congolense* IL 2642 and *T.congolense* IL 2642 1R were tested for their sensitivity to diminazene aceturate, the diminazene aceturate CD_{50} value was shown to have increased by about 2.2-fold from 8.2 (CI: 3.53 – 12.8) to 18.02 mg/kg bw (CI: 5.52 – 30.52). The survival probabilities that infected mice remained infected after treatment with 20 mg/kg bw diminazene were analysed. The results showed that only 2% (95% CI: 0.00 – 0.16) of the mice infected with the parental clone (IL 2642) survived treatment (showed relapse of infection). In contrast, about 47% (95% CI: 0.18 – 0.69) of the mice infected with the isometamidium resistant derivative (IL 2642 1R) survived treatment (showed relapse of infections) when treated with 20 mg/kg bw diminazene (Table 15).

Table 15: Comparison of the isometamidium and diminazene CD_{50} value of *T. congolense* IL 2642 and its isometamidium-resistant derivative IL 2642 1R

	CD_{50} (mg/kg bw)§	**Survival probability (P)†**
Isometamidium		
IL 2642‡	0.0086 (95% CI: - 0.0047 – 0.022)	0.015 (95% CI: 0.00 – 0.076)
IL 2642 1R	1.37 (95% CI: 0.91 – 1.82)	0.68 (95% CI: 0.44 – 0.89)
Diminazene		
IL 2642‡	8.2 (95% CI: 3.53 – 12.8)	0.02 (95% CI: 0.00 – 0.16)
IL 2642 1R	18.02 (95% CI: 5.52 – 30.52)	0.47 (95% CI: 0.18 – 0.69)

§ the dose of drug required to cure approximately 50% of the animals treated
‡ reference sensitive clone
† probability that infected mice remained infected after treatment with 1 mg/kg bw isometamidium or 20 mg/kg bw diminazene (the probability that mice will be cured at this dose is 1-P)

Figure 3 shows the comparison of 'survival curves' of IL 2642 and IL 2642 1R in response to treatment with isometamidium chloride. For any concentration of isometamidium chloride, the rate of decline in 'survivability' (elimination of parasitaemia) was slowest in IL 2642 1R with predicted CD_{50} of 1.37 (95% CI: 0.91 to 1.82) mg/kg bw. The fastest decline was observed in IL 2642 with a predicted CD_{50} of 0.0086 (95% CI: -0.0047 to 0.022) mg/kg bw.

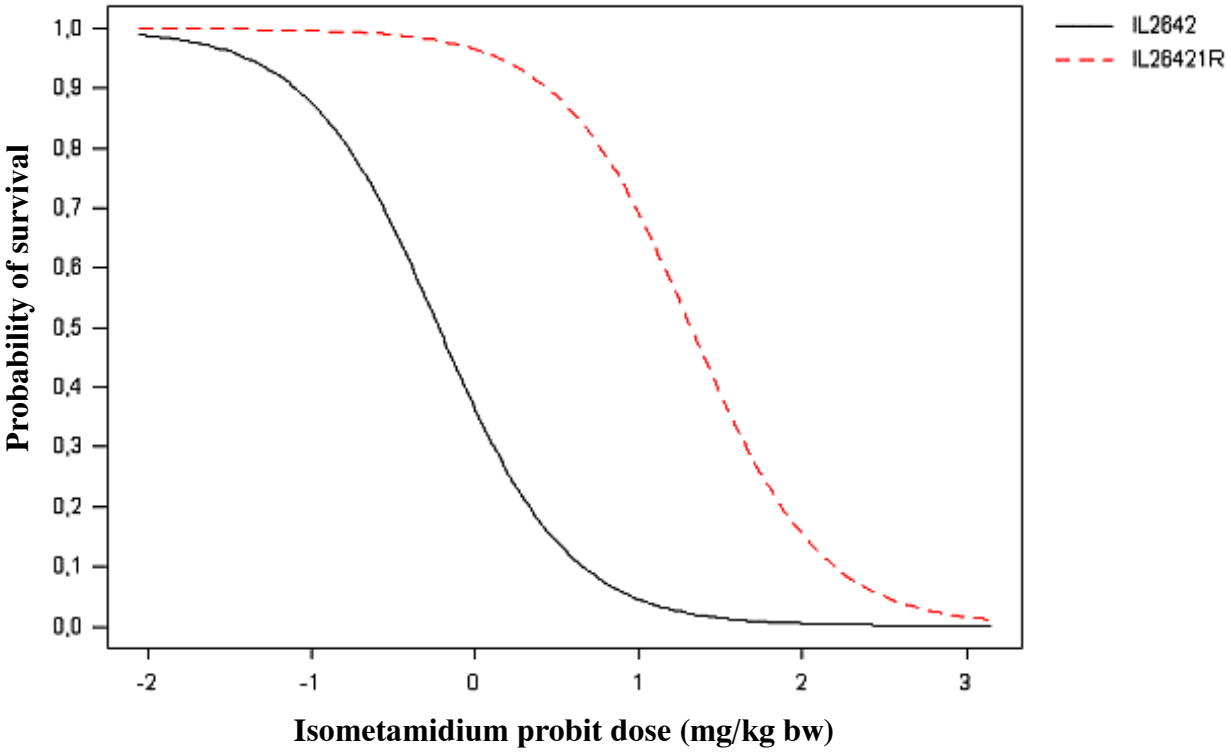

Figure 3: Parametric survival plots of infections with IL 2642 parent and its isometamidium-resistant derivative IL 2642 1R in response to treatment with different doses of isometamidium in mice. The probabilities at which infections were cured are calculated as 1-probability of survival of trypanosomes.

Figure 4 shows the relationship between the predicted probability of 'survival curves' of Il 2642 and IL 2642 1R in response to treatment with diminazene aceturate. At different dosages, the rate of decline in probability of surviving (elimination of the parasitaemia) was slowest with IL 1R. The two curves were quite parallel between 0 and 30 mg/kg bw. But at the highest dosage level the lines converged. The predicted CD_{50} for Il 2642 was 8.2 (95% CI: 3.53 to 12.8) mg/kg bw, whereas the CD_{50} for IL 2642 1 R was 18.02 (95% CI: 5.52 to 30.52) mg/kg bw.

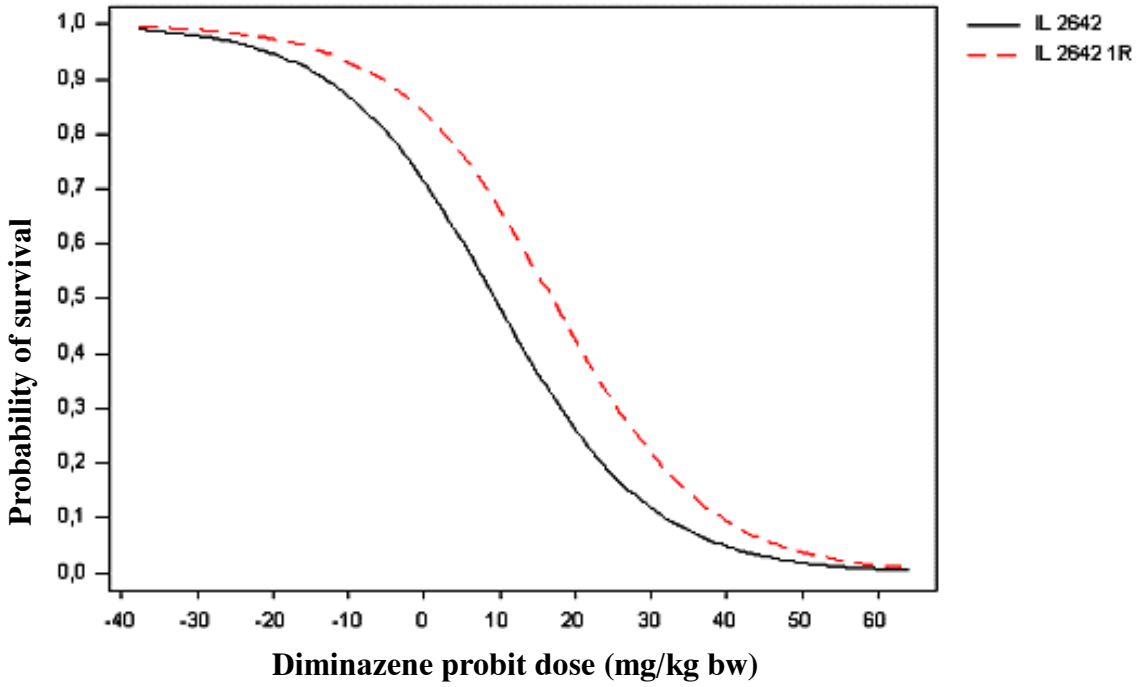

Figure 4: Parametric survival plot of infections with IL 2642 and IL 2642 1R in response to treatment with different doses of diminazene in mice. The predicted probabilities at which infections were cured were calculated as 1-probability of survival of trypanosomes.

5.5 Molecular characterisation of genetic identity of the *T. brucei* and *T. congolense* study clones

5.5.1 T. brucei

Polymerase chain reaction (PCR) assays were used to amplify the *T. brucei* isolates used in this study. The DNA extracted from all the *T. brucei* isolates were successfully amplified using the nuclear repeat primers specific for *Trypanozoon* described in Moser et al. (1989). Consequently, the 177 bp PCR products were detectable when run in 2% gel electrophoresis (Figure 5)

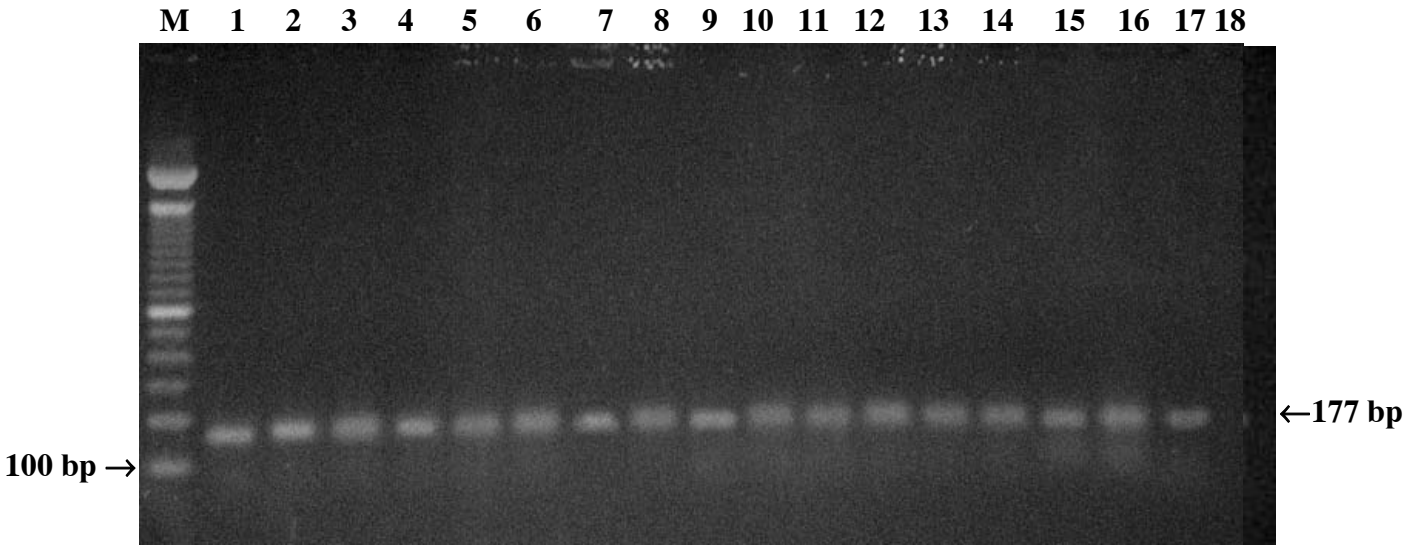

Figure 5: PCR products of a 177 bp fragment of *T. brucei* study stocks using nuclear repeat primers specific to *T. brucei*. M, 100 bp molecular size marker; Lane 1, ILTat 1.4; Lane 2, STIB 345; Lane 3 – 13, *T. brucei* Study stocks (Table 3); Lane 14, CP 547; Lane 15, CP 2469; Lane 16, IG 2602 (*T. b. gambiense*); Lane 17, +ve control; Lane 18, -ve control.

The DNA extracted from the same isolates was further subjected to PCR using the primers described for the SRA gene fragment of *T. b. rhodesiense* (Radwanska et al., 2002). The results showed that no PCR signals were detected except for the *T .b. rhodesiense* positive controls (Figure 6). This suggested that the *T. brucei* isolates used in the current study were all *T. b. brucei.*

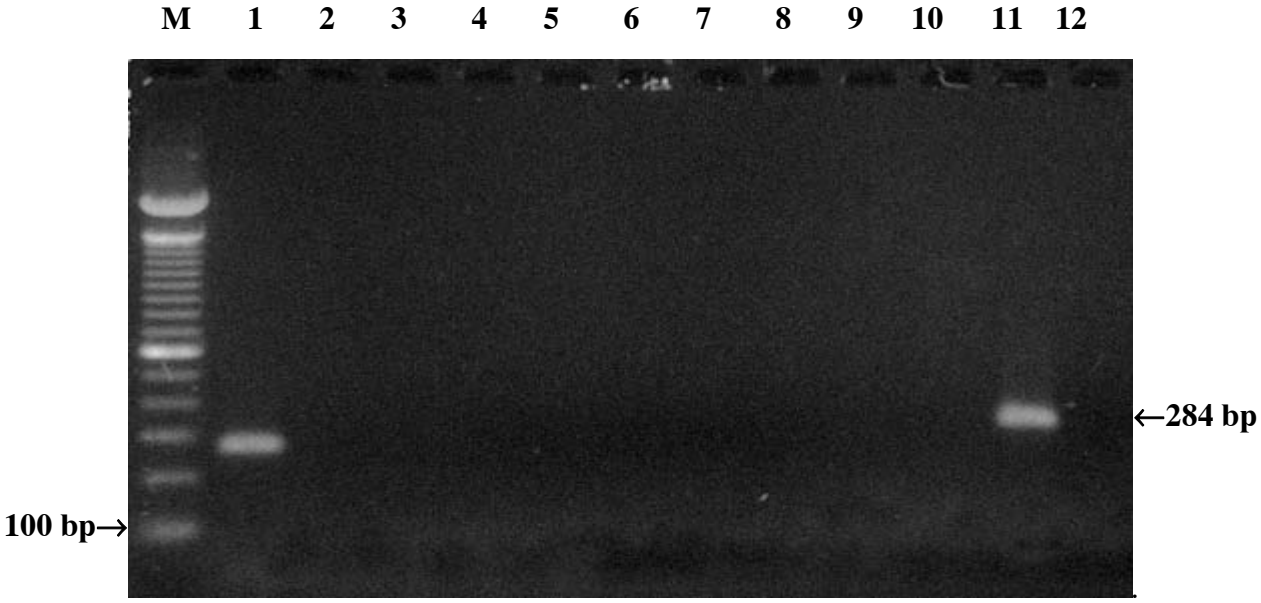

Figure 6: PCR amplification of the SRA gene fragment from the DNA of *T. brucei*. M, 100 bp molecular size marker; Lane 1, IG 2602 (*T. b. rhodesiense*); Lanes 2-10, UG 95 0220, UG 95 0405, UG 95 0505, UG 95 0525, UG 95 0716, UG 95 1017, UG 95 2223, UG 95 2507, UG 95 2821; Lane 11, +ve control (*T. b. rhodesiense*; Lane 12, -ve control.

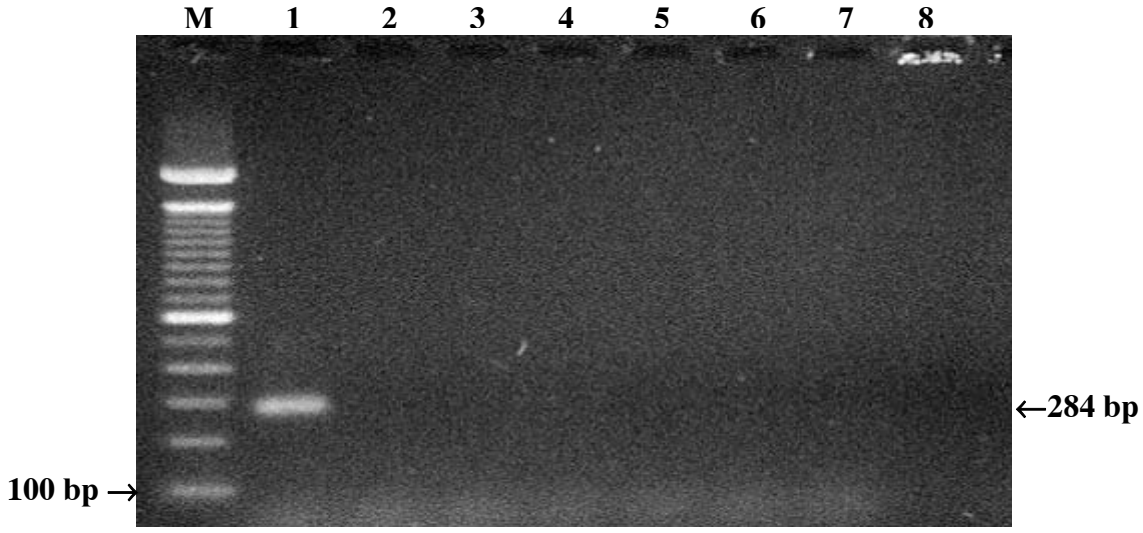

Figure 7: PCR amplification of the SRA gene fragment from the DNA of *T. brucei*. M, 100 bp molecular size marker; Lane 1, IG 2602 (*T. b. gambiense*); Lanes 2, UG 95 5323; Lane 3, UG 95 16323; Lane 4, ILTat 1.4; Lane 5, STIB 345; Lane 6, CP 547; Lane 7, CP 2469; Lane 8, -ve control.

5.5.2 *T. congolense*

The *T. congolense* clones used for the isometamidium sensitivity study were characterised molecularly for their genetic identity. PCR analysis for the DNA-extracts from the *T. congolense* clones, using oligonucletide primers specific for *T. congolense* Savannah (Moser et al., 1989a) and *T. congolense* Forest (Masiga et al., 1992) showed that all the isometamidium resistant clones from Ethiopia and Burkina Faso were savannah types (Figure 8). The 326 bp specific DNA products of *T. congolense* Savannah were successfully amplified in all of the samples examined. DNA extracts derived from the same clones did not produce PCR signals when subjected to the *T. congolense* Forest primers.

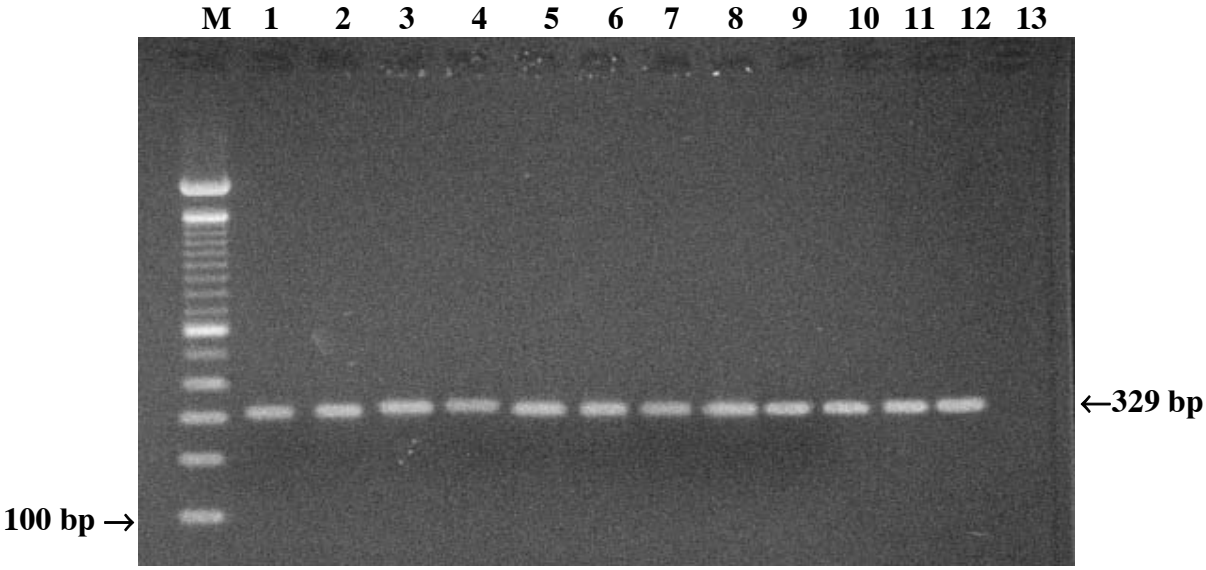

Figure 8: PCR profile of a 326 bp specific DNA product of *T. congolense* Savannah. M, 100 bp molecular size marker; Lane 1, IL 2642; Lane 2, IL 1180; Lane 3, PA 73 clone 1; Lane 4, PA 77 clone 1; Lane 5, PA 77 clone 2; Lane 6, PA 77 clone 3, Lane 7, SA 095 clone 2; Lane 8, SA 095 clone 3; Lane 9, SA 267 clone 1; Lane 10, SA 267 clone 2; Lane 11, SA 278 clone 1; Lane 12, +ve control; Lane 13, –ve control.

5.6 Detection of a fragment of TbAT1 gene in *T. b. brucei* stocks by PCR

Attempts were made to amplify a fragment of the TbAT1 from the DNA of the *T. b. brucei* study stocks, using the primers Sfa-s and Sfa-as (Mäser et al., 1999). The results of PCR analyses showed that 677 bp fragments of the gene, which lie between nucleotides 430 and 1108, were successfully amplified from the genomic DNA of each of the phenotypically

characterised isometamidium-sensitive and -resistant *T. b. brucei* field and laboratory stocks (Figure 9 and 10).

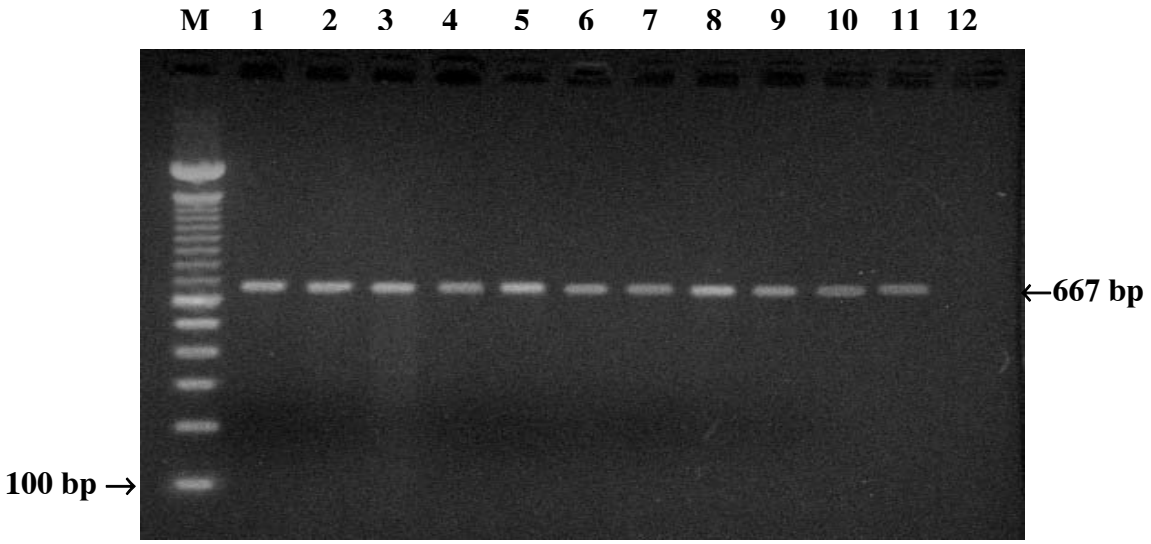

Figure 9: PCR profile of a 677 bp fragment of TbAT1 from the genomic DNA of *T. b. brucei*. M is 100 bp molecular size marker; Lane 1, ILTat 1.4; Lanes 2-10, UG 95 0220, UG 95 0405, UG 95 0505, UG 95 0525, UG 95 0716, UG 95 1017, UG 95 2223, UG 95 2507, UG 95 2821; Lane 11, STIB 345; Lane 12, -ve control.

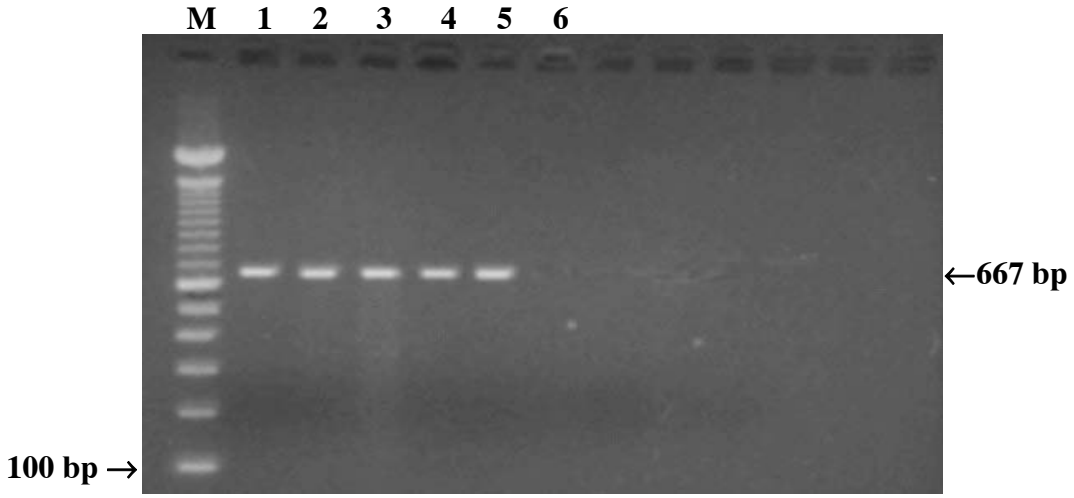

Figure 10: PCR profile of a 677 bp fragment of TbAT1 from the genomic DNA of *T. b. brucei*. M is 100 bp molecular size markers; Lane 1, STIB 345; Lanes 2, UG 95 53 23; Lane 3, UG 95 163 23; Lane 4, CP 547; Lane 5, CP 2469; Lane 6, -ve control.

5.7 Sequence analyses of the TbAT1 gene fragments from the genomic DNA of isometamidium-sensitive and -resistant *T. b. brucei* stocks

The nucleotide sequences of the 677 bp central fragment of the TbATl gene (nucleotides 430-1108) from isometamidium-sensitive *T. b. brucei* stocks were aligned with those from isometamidium-resistant stocks and reference sequences for wild-type and mutant-type *T. b. brucei* (GenBank accession numbers AF 152369 and AF 152370, respectively) (Figure 11). The results indicate that all the *T. b. brucei* stocks in this study, which were phenotypically characterised as isometamidium-sensitive, contained the wild-type sequence patterns. In contrast, the isometamidium-resistant *T. b. brucei* stocks (CP 547 and CP 2469) showed sequence patterns that corresponded to the DNA sequence of the laboratory-derived melarsoprol-resistant STIB 777R stock. Summary of the nucleotide variations detected in the diagnostic positions of the 677 bp TbAT1 gene sequence alignment among the different stocks are reported in Table 16. Both nucleotide sequences from the isometamidium-resistant *T. b. brucei* showed the same set of six mutations detected in the laboratory-derived melarsoprol-resistant stock. These differences were as follows: substitution of C by T at nucleotide 471, G by A at nucleotide 532, G by A at nucleotide 542, A by G at nucleotide 716, A by G at nucleotide 857 and C by T at nucleotide 1008. Four of these nucleotide differences detected in the 677 bp gene fragments led to amino acid substitutions: $Ala^{178} \rightarrow$ Thr (A178T), $Gly^{181} \rightarrow$ Glu (Gl181E), $Asp^{239} \rightarrow$ Gly (D239G) and $Asn^{286} \rightarrow$ Ser (N286S). Furthermore, deletions of three nucleotides (TTC), which encode the amino acid phenaylalanine, were detected at nucleotide positions 949, 950 and 951 of both of the resistant stocks.

Results of the analysis of the restriction sites of the 677 bp TbAT1 gene fragments revealed that all isometamidium-sensitive *T. b. brucei* field stocks and both of the isometamidium-sensitive reference stocks (STIB 345 and ILTaT 1.4) consisted of similar Sfa NI restriction sites. Whereas, the isometamidium-resistant reference *T. b. brucei* stocks (CP 547 and CP 2469) showed Sfa NI restriction sites different from those of the sensitive ones (Figure 11). The point mutation that led to the substitution of the nucleotide G by A at nucleotide position 532 (G532A) in the sensitive stock abrogated the Sfa NI restriction site. In contrast, the mutation at 857 bp that led to the substitution of A by G (A857G) resulted in a new Sfa NI restriction site. This mutation in the isometamidium-resistant *T. b. brucei* stock resulted in the shift of Sfa NI restriction sites by 323 bp positions further downstream.

***T. b. brucei* stocks**

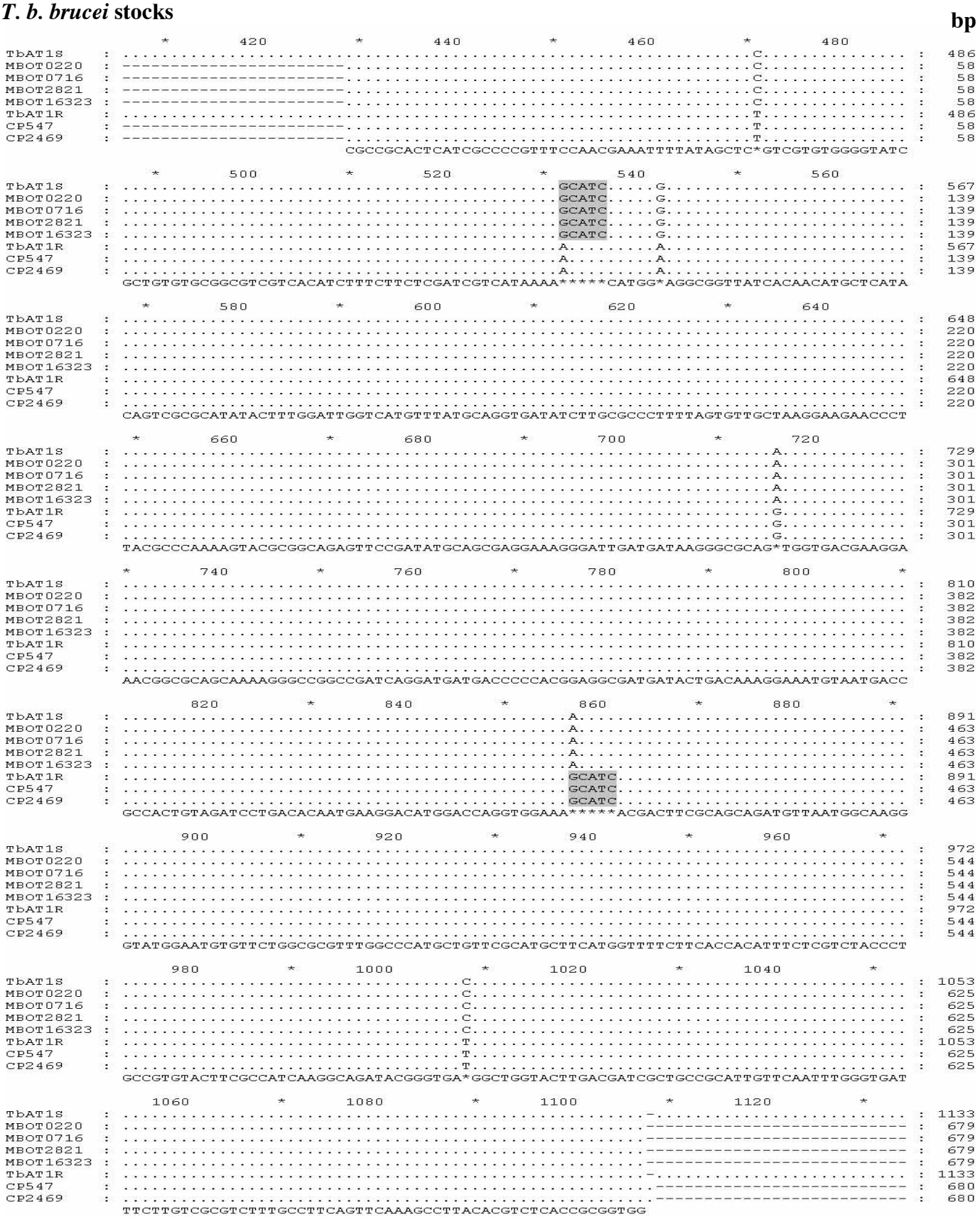

Figure 11: Nucleotide sequence alignments of the TbAT1 gene fragment in isometamidium-sensitive and -resistant *T. b. brucei* stocks. Identical sequences are shown by dots (.) and differences by letters representing nucleotides. Sfa NI restriction sites are shaded in dark. TbAT1S and TbAT1R refer to the nucleotide sequences in the wild-type and mutant-type TbATl, respectively (GenBank accession numbers AF152369 and AF152370, respectively).

T. b. brucei stocks

bp

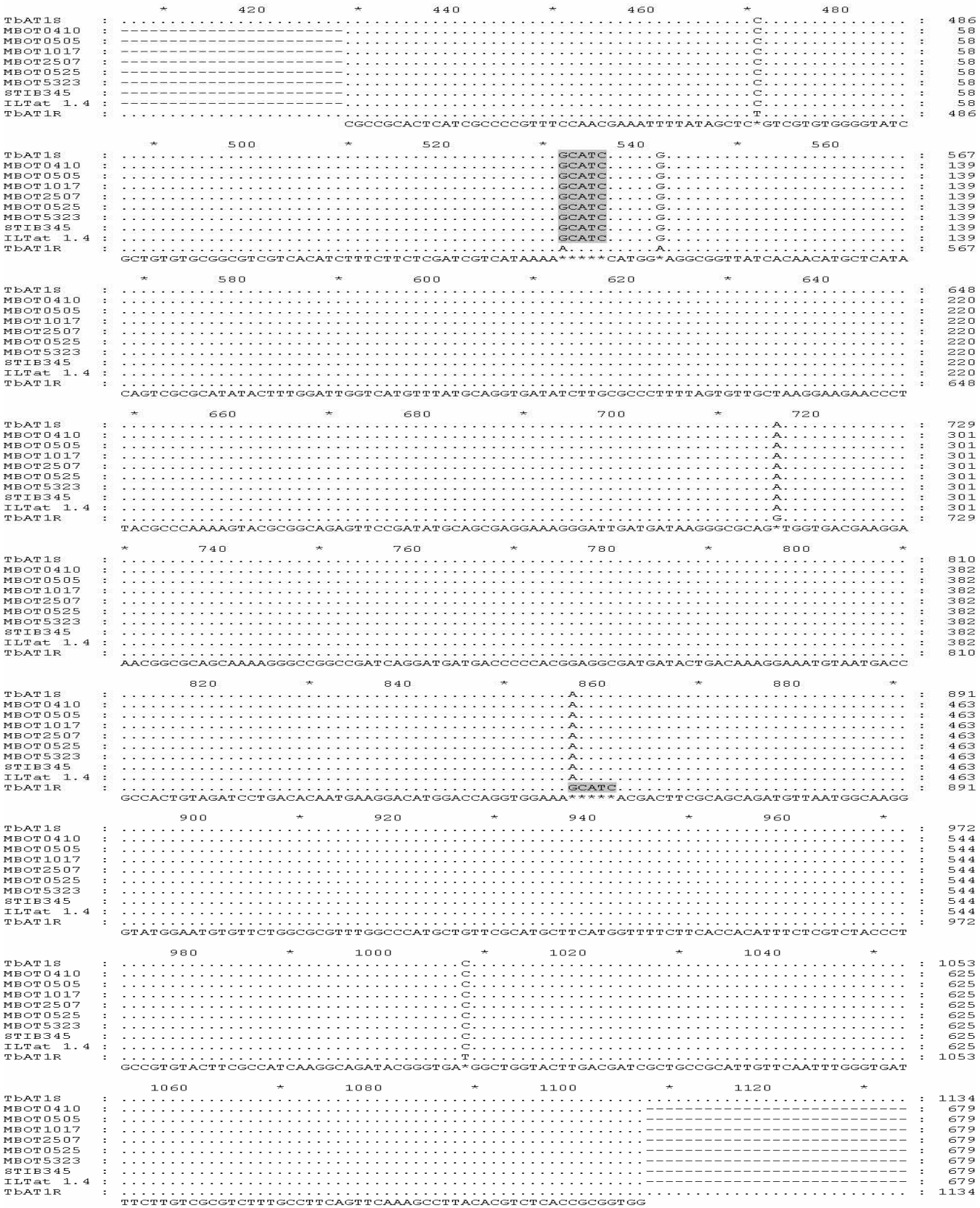

Figure 12: Nucleotide sequence alignments of the TbAT1 gene fragments of isometamidium-sensitive *T. b. brucei* stocks. Identical sequences are shown by dots (.) and differences by letters representing nucleotides. Sfa NI restriction sites are shaded in dark. TbAT1S refer to the nucleotide sequence for the wild-typeTbATl (GenBank accession number AF152369).

Table 16: Sumary of the nucleotide variations detected in the 677 bp gene fragment (nucleotides 430-1108) of the TbAT1 gene between isometamidium-sensitive and -resistant *T. b. brucei*

***T. b. brucei* Isolates**	**ISMM sensitivity Phenotype**	**Alignment Positions**					
		471	532[aa, §]	542[aa]	716[aa]	857[aa, §]	1008
TbAT1[S]*	S	C	**G**	G	A	**A**	C
STIB 345	S	C	**G**	G	A	**A**	C
ILTAT 1.4	S	C	**G**	G	A	**A**	C
MBOT/ UG/95 0220	S	C	**G**	G	A	**A**	C
MBOT/UG/95 0410	S	C	**G**	G	A	**A**	C
MBOT/UG/95 0505	S	C	**G**	G	A	**A**	C
MBOT/UG/95 05 25	S	C	**G**	G	A	**A**	C
MBOT/UG/95 0716	S	C	**G**	G	A	**A**	C
MBOT/UG/95 1017	S	C	**G**	G	A	**A**	C
MBOT/UG/95 22 23	S	C	**G**	G	A	**A**	C
MBOT/UG/95 25 07	S	C	**G**	G	A	**A**	C
MBOT/UG/95 28 21	S	C	**G**	G	A	**A**	C
MBOT/UG/95 53 23	S	C	**G**	G	A	**A**	C
MBOT/UG/95 163 23	S	C	**G**	G	A	**A**	C
TbAT1[r]*	R	T	**A**	A	G	**G**	T
CP 547	R	T	**A**	A	G	**G**	T
CP 2469	R	T	**A**	A	G	**G**	T

ISMM, isometamidium; S, sensitive; R, resistant; TbAT1s*, melarsoprol-sensitive reference stock 777S (GenBank accession number AF152369); TbAT1[r]*, melarsoprol-resistant reference stock 777R (GenBank accession number AF152370); aa, nucleotide differences manifested at the amino acid level; §, position of point mutation that resulted in the Sfa NI RFLP; A, Adenine; C, Cytosine; G, Guanine; T, Thymine.

5.8 Sfa NI RFLP analysis

In order to analyse the RFLP pattern of a fragment of TbAT1 (nucleotides 430-1108), the 677 bp PCR products from 8 of the isometamidium-sensitive and 2 of the isometamidium-resistant *T. b. brucei* were subjected to digestion with Sfa NI and analysed on a 2% agarose gel. The results revealed two different banding patterns: The digest resulted in fragment sizes of 566 and 111 bp in the case of TbAT1 from isometamidium-sensitive stocks and fragment sizes of 435 and 242 bp in the case of TbAT1 from isometamidium-resistant stocks (Figures 13 and 14).

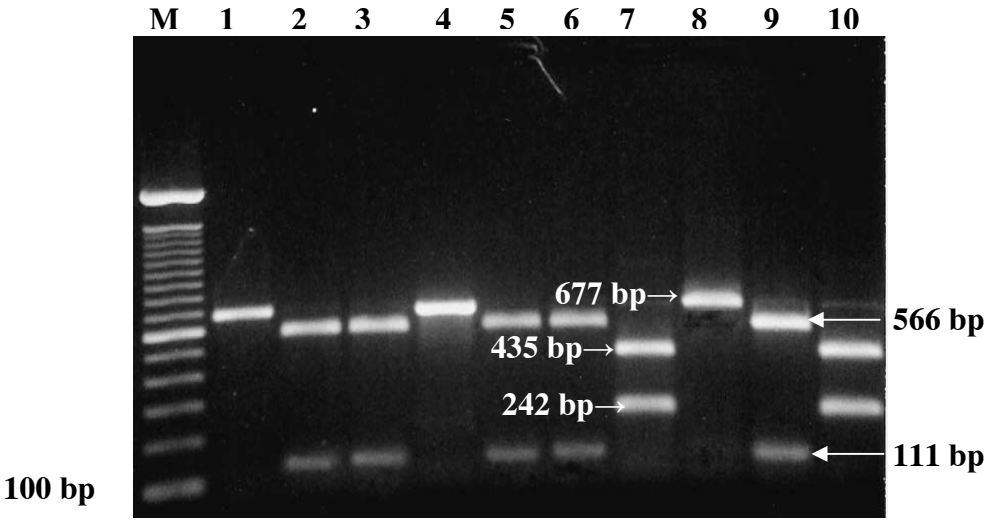

Figure 13: Restriction profiles of a fragment of the TbAT1 (nucleotides 430-1108) from isometamidium-sensitive and -resistant *T. b. brucei* stocks digested with Sfa NI. A diagnostic digest with Sfa NI produces fragment sizes of 566 and 111 bp in the case of TbAT1 from isometamidium-sensitive stocks and 435 and 242 bp in the case of TbAT1 from the resistant stocks. M, 100 bp molecular size Marker; Lane 1, MBOT 163 23 undigested; Lane 2, MBOT 163 23 digested; Lane 3, MBOT 2507, digested; Lane 4, MBOT 0220 undigested; Lane 5, MBOT 0220 digested; Lane 6, MBOT 2507 digested; Lane 7, CP 547 digested; Lane 8, CP 547 undigested; Lane 9, MBOT 0505 digested and Lane 10, CP 2469 digested.

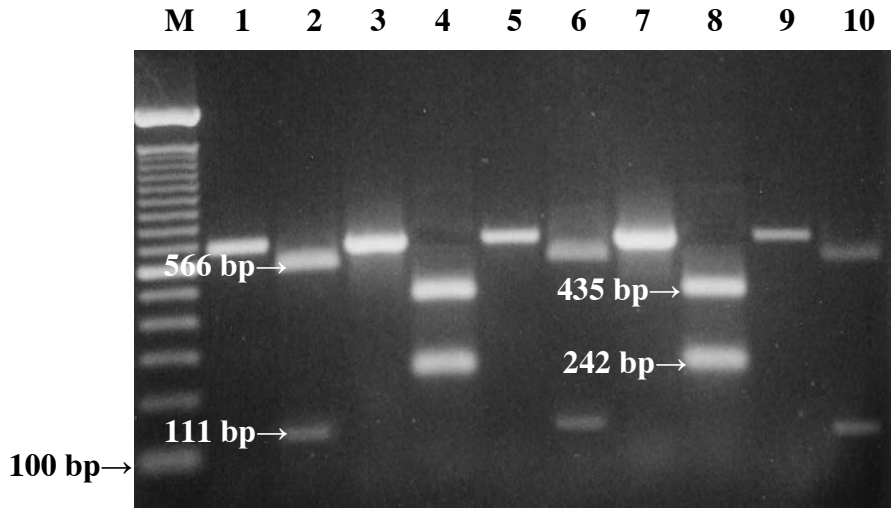

Figure 14: Restriction profiles of a fragment of TbAT1 (nucleotides 430-1108) from isometamidium-sensitive and -resistant *T. b. brucei* stocks digested with Sfa NI. A diagnostic digest with Sfa NI produces fragment sizes of 566 and 111 bp in the case of TbAT1 from isometamidium-sensitive stocks and 435 and 242 bp in the case of TbAT1 from the resistant stocks. M, 100 bp molecular size Marker; Lane 1, MBOT ILTat 1.4 undigested; Lane 2, ILTaT 1.4 digested; Lane 3, CP 547, undigested; Lane 4, CP 547 digested; Lane 5, MBOT 0410 undigested; Lane 6, MBOT 0410 digested; Lane 7, CP 2469 undigested; Lane 8, CP 2469 digested; Lane 9, MBOT 1017 undigested; Lane 10, MBOT 1017 digested.

5.9 Characterisation of putative target sequences in *T. congolense* homologues to *T. brucei* adenosine transporter genes (TbAT1)

5.9.1 PCR analysis of the genomic DNA of *T. congolense* for the presence of the 677 bp TbAT1 transcripts

PCR amplification of the 677 bp fragment of the TbAT1 gene sequence described in *T. brucei* from the genomic DNA of *T. congolense* clones was performed. The oligonucleotide primer sets described for the TbAT1 gene fragment, sfa-s 5´-CGCCGCACTCATCGCCCCGTTT-3´ and sfa-as 5´-CCACCGCGGTGAGACGTGTA-3´, were used. Clones of *T. congolense* from East and West Africa were analysed using this pair of primers. The results showed that the TbAT1 specific primer pair did not yield any detectable PCR products when used on the DNA of *T. congolense* tested (Figure 15). In contrast, the gene was detected in the DNA of the *T. b. brucei* reference clones, STIB 345 and ILTat 1.4.

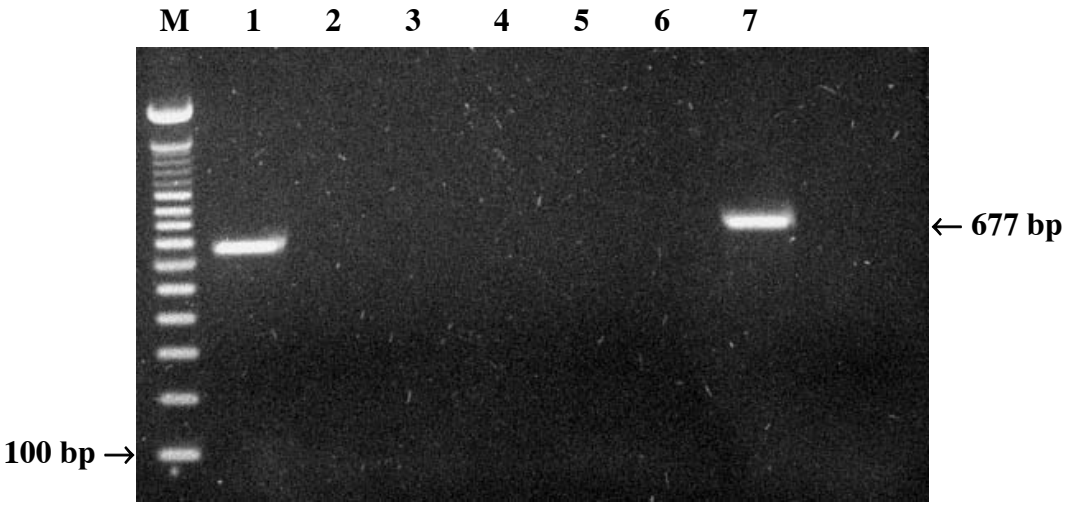

Figure 15: PCR analyses of genomic DNA of *T. congolense* with Sfa-s and Sfa-as primers. M is 100 bp molecular size markers, Lane 1, ILTaT 1.4 (+ve control); Lane 2, IL 1180; Lane 3, IL 2642; Lane 4, PA 073 clone 1; Lane 5, SA 95 clone 2; Lane 6, SA 268 Clone 1; Lane 7, STIB 345 (+ve control); Lane 8, -ve control

5.9.2 Investigation of TbAT1 homologous gene transcripts in *T. congolense* using sets of primers flanking different regions of the gene

The nucleotide sequence data of the TbAT1 gene transcript of *T. b. brucei* was accessed from the GenBank (accession number AF 152369). The sequence information was used to design four pairs of primers that flank different locations of the gene. The pairs of primers were selected and picked by using the computer program Primer3 (htpp://frodo.wi.mit.edu) (Rozen and Skaletsky, 2000). These pairs were used to search for homologous gene sequences in the genomic DNA of *T. congolense* by PCR. While no PCR product was detected from the DNAs of the *T. congolense*, all the expected PCR products could be detected from the DNAs of the *T. b. brucei* positive controls (Figures 16 and 17).

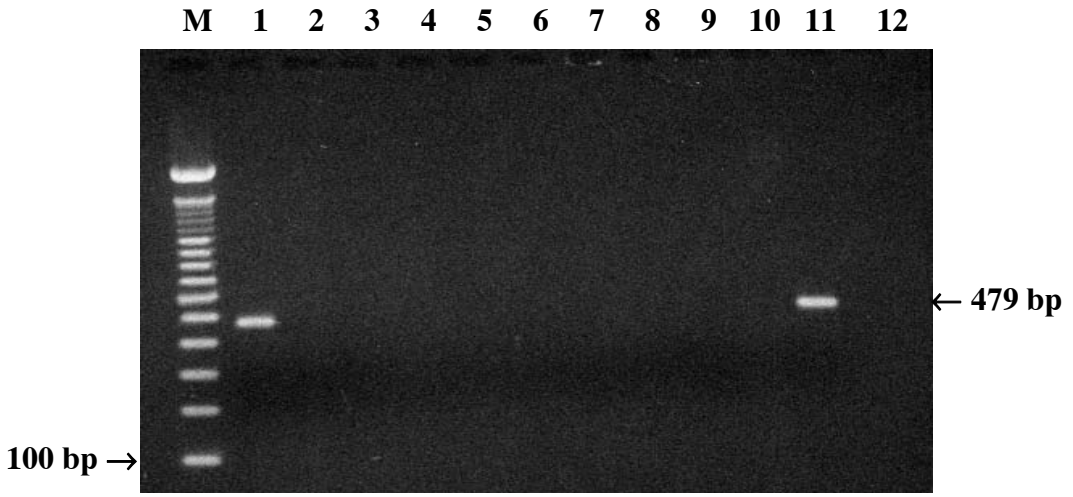

Figure 16: PCR analyses of genomic DNA of *T. congolense* using Sfa1-s and Sfa1-as primers. M, 100 bp molecular size marker, Lane 1, ILTAT 1.4; Lane 2, IL 2642; Lane 3, IL 2642; Lane 4, PA 073 clone 1; Lane 5, PA 77 clone 1; Lane 6, PA 77 Clone 2; Lane 7, SA 95 clone 2; Lane 8, SA 95 clone 3; Lane 9, SA 267 clone 1; Lane 10, SA 268 clone 2; Lane 11, STIB 345; Lane 12, -ve control

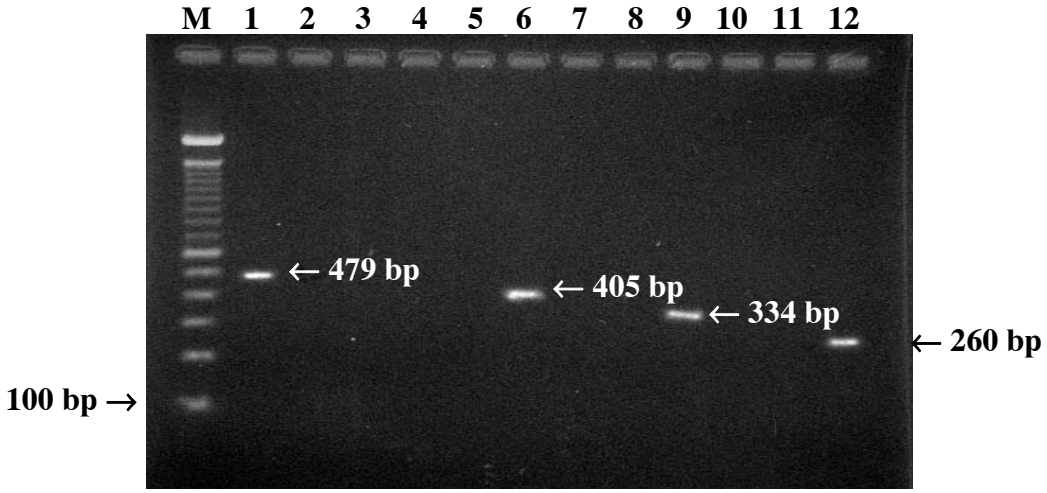

Figure 17: PCR analyses of *T. congolense* using four pair of primer sets flancking to different regions of TbAT1. M, 100-bp DNA marker, Lane 1, STIB 345; Lane 2, IL 1180; Lane 3, IL 2642; Lane 4, PA 73 clone 1; Lane 5, PA 77 clone 1; Lane 6, ILTat 1.4; Lane 7, IL 1180; Lane 8, SA 95 clone 2; Lane 9, STIB 345; Lane 10, IL 2642; Lane 11, SA 267 clone 1; Lane 12, ILtat 1.4.

Table 17: Results of PCR analyses of trypanosome stocks for TbAT1 gene using different primer sets

Lane	DNA sample	Expected PCR product size	Result
1	STIB 345	479	Positive
2	IL 1180	479	Negative
3	IL 2642	479	Negative
4	PA 73 clone 1	405	Negative
5	PA 77 clone 1	405	Negative
6	ILTat 1.4	405	Positive
7	IL 1180	334	Negative
8	SA 95 clone 2	334	Negative
9	STIB 345	334	Positive
10	IL 2642	260	Negative
11	SA 267 clone 1	260	Negative
12	ILTat 1.4	260	Positive

5.9.3 Genomic DNA analysis of *T. congolense* for the presence of TbAT1 homologous sequences using degenerate primers

Three pairs of degenerate primers were generated by doing Global DNA alignments of the gene sequences of *T. b. evansi* and *T. b. equiperdum* against the reference molecule (the TbAT1 nucleotide sequence in *T. b. brucei*). The primer sets generated were used to attempt the amplification of the respective genes in the DNA of *T. congolense* IL 1180, IL 2642 and ILTat 1.4 by gradient PCR. The results (Table 18) showed that none of the degenerate primers produced specific PCR products when used on the DNA of the *T. congolense.* Nevertheless, detectable bands were observed in the case of the *T. b. brucei* ILTat 1.4 reference clone.

Table 18: Results of PCR analyses of trypanosome stocks for TbAT1 gene using degenerate primers

Degenerate primer sets	**Expected PCR product size (bp)**	**DNA sample**	**Result**	**Annealing Temperature**
TBAT1 FW &TBAT2 RV	867	STIB 345	Positive	52.8 and 57.4^{0}C
		IL 1180	Negative	
		IL 2642	Negative	
TBAT3 FW & TBAT7 RV	698	STIB 347	Positive	52.8 and 57.4^{0}C
		IL 1180	Negative	
		IL 2642	Negative	
TBAT1 FW & TBAT7 RV	750	STIB 345	Positive	52.8 and 57.4^{0}C
		IL 1180	Negative	
		IL 2642	Negative	