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Comparison of drug Sensitivity and Pathogenicity of *Trypanosoma brucei rhodesiense* isolates with their respective clones originating from Busia and Busoga.

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Abstract

Introduction: Studies using clones in the laboratory show variations in pathogenicity and drug sensitivity compared to their respective natural isolates for various trypanosome species. This suggests that within isolates there exist sub-populations due to change overtime. This study characterized *Trypanosoma brucei rhodesiense* isolated from patients in two different sleeping sickness foci, (Busoga and Busia) and evaluated the change in drug sensitivity and pathogenicity with time of isolation.

Methods: In the study, various *Trypanosoma brucei rhodesiense* clones and their respective isolates were tested for pathogenicity and sensitivity to drugs (Suramin and Mel B) in Swiss white mice. The clinical, pathological and sensitivity parameters were determined.

Results: When compared with the specific isolates, clones had lower mean pre-patent periods and lower mean post infection survival times. Furthermore, when compared with the controls, packed cell volume (PCV) changes for all the clones were significantly different ($p<0.05$) but this was not the case for the isolates.

Conclusion: The study concluded that the clones were more pathogenic than their isolates. Drug sensitivity results for various isolates and their respective clones were comparable at all dosages except for EATRO 1886 isolate which was found to be resistant to 2.5mg/kg of Suramin.

Key words: Isolates, *Trypanosome brucei rhodesiense,* Clones, Melarsoprol, drug sensitivity,

Introduction

Human African Trypanosomosis (HAT) is a re-emerging disease caused by the flagellated parasites; *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei*. *gambiense*, occurring in 36 African countries (WHO, 2001). It is estimated that 60 million people living in 250 foci are at risk of contracting the disease with 500,000 new cases reported annually (WHO, 2005). In East Africa, Busoga and Busia are the most affected regions with human infective forms of the disease (Legros *et al.,* 1999a).

Busoga is found in the south eastern part of Uganda where an epidemic of a more acute severe sleeping sickness was first discovered in 1898 and it normally experiences massive epidemics (Picozzi *et al.*, 2005). Wars, insecurity, civil unrest and deteriorating economic circumstances led to a breakdown of health services and disease control, contributing to the frequent epidemics occurring in the area (Louis, 2000). With these factors, epidemics finally spread to neighboring countries and districts. On the other hand, Busia experienced epidemics of sleeping sickness in 1940s and in 1960s. However, few cases are being reported due to the active surveillance programs that are going on over the last 30 years (Jannin *et al*., 2001).

Faced with continued lack of vaccine which is far from being successful, (Delespaux and dekoning, 2007) chemotherapy remains the chief support for the disease control in the future (Keiser *et al.,* 2001). Studies have shown that frequent exposure of drugs in the field promotes mutation among the parasites leading to drug resistance (Kagira and Maina, 2007). Chemotherapy relies mainly on handful of drugs, (Pepin and Milford, 1994). However, these drugs have been faced with resistance (Geerts *et al.,* 2001) which is a major cause of treatment failures reported (Balasegaram *et al*., 2006).They are also associated with adverse side effects (Lejon *et al.,* 2003). These studies have shown an alarming rate of development of drug resistance among trypanosomes (Kennedy, 2007) thus calls for proper use of the few existing drugs to limit development of resistance.

Trypanosome variant surface glycoprotein (VSG) genes are of great importance in pathogenicity of trypanosomes since they provide molecular basis for the antigenic variation during an infection (Inverso *et al.,*

2010). The frequent changing of the VSG leads to the production of a wave of parasitaemia that characterizes the disease and also produces various sub populations in an isolate (Mutugi *et al.,* 1995). VSG molecule has been associated with virulence, and studies have shown that parent cell trypanosome expressed a surface coat distinct from each of the virulent daughter cells (Magez *et al.,* 1998). Other results gave evidence that clones and sub clones were different in virulence. Magez *et al.,*(1997). Therefore, it was suggested that the VSG molecule could play a direct role in the relative expression of trypanosome virulence expressed in a host (Lucas *et al*., 1994). In that regard, the present study determined the effect of cloning *T. b. rhodesiense* isolate on sensitivity (suramin and melarsoprol) and pathogenicity.

2.0 Materials and Methods

All experimental procedures and protocols involving mice were reviewed and approved by Institutional Animal care and Use Committee (IACUC) of Trypanosomosis Research Centre (TRC) Muguga, Kenya.

2.1 Trypanosome isolate and clones used

Two *Trypanosoma brucei rhodesiense* isolates from Busoga (Uganda) and other two from Busia, (Kenya) with their respective clones were used for experiment in this study (Table 1). These clones were developed from the isolates as described by Van Meirvenne *et al.,* (1975) and Gardiner *et al.,* (1980). They had been cryopreserved at the KARI-TRC trypanosome bank in liquid nitrogen at -196°C

2.2 Laboratory animals

Swiss White mice weighing between 25-30g from Trypanosomosis Research Centre Animal Breeding Unit (Kenya) were used. Each of the eight stabilates required 32 mice, making a total of 256 mice for the whole experiment. They were housed in cages with saw dust as bedding material and fed on commercial pellets (Kenya Unga ® Ltd) and water provided *ad libitum.*

2.3 Experimental design

Experimental design for marking and immunosupression, infection, drug preparation and treatment are described below.

2.3.1 Marking and immunosuppression of mice

A total of 32 mice (30 experimental and 2 donors) for each of the eight stabilates from Busia and Busoga were marked using picric acid. Experimental mice were divided into four groups of five mice each and the fifth group had ten mice. The two donor mice for each stabilate were immunosuppressed using cyclophosphamide at a concentration of 200mg/kg body weights for three consecutive days before infection (Kagira *et al.,* 2005).

2.3.2 Infection of donor and experimental mice

Trypanosome stabilates stored in the trypanosome bank at -196°C in liquid nitrogen in small capillary tubes of 20µl each, were brought in a cryovial. Viability of the parasites in the capillary tube was checked under a microscope. Thawed EDTA saline glucose (ESG) buffer was used to dilute the parasites then 0.2ml of the dilution was intraperitoneally injected to each donor mouse.

Follow up of the parasitaemia of the donor mice was done, and on the first peak of the parasitaemia (Kagira *et al.,* 2005), a few drops of blood was taken from the tail of one of the donor mouse and mixed with 2 ml of ESG buffer in a bijou bottle. This blood solution was diluted 10 times with ESG buffer using a leukopipette. The number of trypanosomes was estimated using an improved Neubauer chamber viewed under the microscope at 400x magnification. The 1st and the 2nd count of the parasites (C_1 and C_2 respectively) were made through all the 16 squares of the haemocytometer and the average count C_{av} calculated. Using the formulae below the number of trypanosomes for infection was quantified.

No. of trypanosomes = Average count x dilution factor x 10^4 trypanosomes/ml.

Serial dilution was done so as to get a solution of 5.0 x 10^4 trypanosomes/ml. To each experimental mouse, 0.2 ml of this dilution was injected intraperitoneally so that each one of them gets 1.0 x 10^4 trypanosomes (Gichuki and Brun, 1999).

2.3.3 Drug preparation and treatment of mice

The drug dosages were calculated as per the standards laid down by World Health Organization (WHO, 2001). For each stabilate, a Suramin dosage of 2.5mg/kg and 5mg/kg and Mel B dosage of 1mg/kg and 20mg/kg were prepared and used for treatment in the respective groups. Group one and two were treated with Suramin at different doses while group three and four were treated with Melarsoprol at different doses too. Group five with ten mice acted as untreated controls. Treatment was done 24 hours after infection as shown in Table 2

2.3.4 Sensitivity and pathogenicity studies

For sensitivity study, treated mice for every stabilate were followed for a period of 60 days post treatment. Blood was taken for parasitaemia examination every other day, for PCV reading after every three days while body weights were taken weekly.

Pathogenicity study was done on the ten infected and untreated mice for all the stabilates. PCV and bodyweight examinations were carried out after every three days (Morton *et al.*, 1993) until the mice died. Mice were examined daily until the parasites were detected in blood so as to get the pre-patent period. After the identification of the pre-patent period, parasitaemia checking was reduced to after every one day to determine its progression.

3.0 RESULTS

3.1 Pathogenicity study

From the table below,(table 3) the mean pre-patent period (ppp) in the infected non treated mice were varying among all the strains, with all the clones having a mean ppp of 3 days which was lower than their respective isolates, except for the clone KETRI 3305, which had a mean ppp of 6 days as compared to a mean ppp of 5 days of the isolate (EATRO 1886). The pre-patent periods of the Busia isolates KETRI 3685 and KETRI 3440 were significantly different ($P<0.001$ and $P=0.004$ respectively) from that of their respective clones. In contrast, there was no significant difference ($P=0.182$ and $P=0.442$ respectively) in the pre-patent periods between the Busoga isolates (EATRO 2285 and EATRO 1886) and their respective clones.

Most mice infected with the clones recorded a lower mean survival period than mice infected with their parent isolates. Mice infected with the Busia clone KETRI 3800 and another clone (KETRI 3380) from a Busoga isolate had the lowest mean survival period of 20 and 21 days respectively while mice infected with the Busoga isolates (EATRO 2285 and EATRO 1886) recorded the highest mean survival time of 47 and 44 respectively with 6 mice and 1 mouse respectively remaining alive at the end of the experiment.

3.2 Packed cell volume (PCV)

The PCV behaved differently with a general reduction in PCVs of mice infected with all the isolates and their clones. There was no significant ($p>0.05$) change in PCV with time of mice infected with the isolates KETRI 3440 and EATRO 1886 and their respective clones (Fig 3.1 and 3.2). However, significant ($p<0.05$) change in PCV with DPI was observed between mice infected with isolates EATRO 2285 and KETRI 3685 and their respective clones. PCV for the clones dropped statistically faster than those for the isolates, as indicated by the fitted regression lines shown below (Fig. 3.3 and 3.4). When the clones and the isolates were compared with the controls, the interaction between the DPI and the clone as compared to the constant (control) for all the clones were significantly different $p<0.05$) while the interaction for the DPI and the isolate for all the isolates showed no significant different ($p>0.05$) results.

3.3 Parasitaemia development

There was significant difference ($P>0.001$) in parasitaemia development for the Busia isolate KETRI 3685 and its respective clone. The average day of the first parasitaemia detection in blood was 3 days for the isolate KETRI 3685 which was significantly lower than that of KETRI 3800 clone (7 days). Starting from the same amount of trypanosomes, parasitaemia development was faster in the isolate KETRI 3685 with a mean parasitaemia of antilog 3.4 on day one post infection than its clone KETRI 3800 which had no parasites detected (Fig. 3.5). No significant difference ($P>0.05$) was recorded in parasitaemia development for other isolates and their clones.

For all the clones and their respective isolates the patterns of parasitaemia were characterized by many waves with no prominent peaks observed. In the same way, the maximum parasitaemia reached in the time course of the experiment was a mean of antilog 8.4 ($2.51x10^8$) trypanosomes for most clones and isolates.

3.4 Sensitivity study

All the mice infected with all the T. b. r. strains were cured by the drug dosages used except for the Busoga isolate (EATRO 1886) which was resistant to the lower dosage of Suramin 2.5mg/kg only, but was cured by other dosages. Relapse to the drug at this dosage occurred on day 17 onwards until the end of the experiment. As shown in the Table 4

Interpretation

The results in the study were interpreted as described by Eisler *et al.*, (2001). A trypanosome isolate was considered as drug sensitive if at least four out of five treated mice were cured. If fewer than four mice were cured, the isolate was considered resistant.

4.0 DISCUSSION

In the present study, clones displayed more pathogenic results than their respective isolates with short mean survival time and short mean ppp. Garba, (1991) obtained similar results in a previous study and thus indicated

that clones rendered the host more susceptible to infection. Clones derived from recently isolated isolates were of high virulence than those clones derived from isolates isolated earlier as observed in KETRI 3800 and KETRI 3380 whose isolates (KETRI 3685 and EATRO 2285) were isolated in the years 2000 and 1976 respectively. They therefore highlighted a high degree of incompatibility as compared to others. KETRI 3380 clone was the most pathogenic and survived for a mean survival time of 21 days while its isolate became chronic with the highest mean survival time of 47 days rendering 7 infected non treated but highly positive mice live by the end of the experimental period. This suggests that trypanosomes genetically evolve with time.

PCV which was found out to be a measure of anaemia (Kagira *et al.,* 2006) recorded a significant difference ($p<0.05$) between KETRI 3685 and EATRO 2285 and their clones (KETRI 3800 and KETRI 3380 respectively). Mice infected with clones recorded a significant drop in PCV and as such died earlier due to anaemia recording the lowest mean survival times of 20 and 21 respectively. Mice infected with the isolates were probably able to control infection and destruction of red blood cells, (Kariuki *et al.,* 2008) a factor which enhanced their survival time. Although PCV for other isolates were not significantly different with their clones, it was found out that PCV for the clones generally declined more as the infection progressed. The mean PCV for the controls (non infected mice) which was maintained at a mean of above 50% was stable throughout the experiment and in comparison with the PCV for the clones, there was a significant difference, ($p<0.05$). Therefore, mice infected with clones became weak and died earlier

No significant difference ($p>0.05$) was observed in parasitaemia development among all the clones and isolates. However, parasitaemia levels of mice infected with clones were generally higher. All the graphs were characterized by many waves but with no prominent peaks. Expression of several waves of parasitaemia is an indicator of different variable antigen types (VATs) of trypanosomes to which immune response is elicited (Brancrof and Askonas, 1985). The rate at which VSG gene switch occurs is very similar to the rate at which the immune system develops an effective antibody response (Turner and Ormerod, 1984). The lower level of parasitaemia demonstrated by mice infected with isolates indicated the ability of the immune system to control the parasite. While the higher parasitaemia observed in the mice infected with clones may suggest that these parasites were more virulent. Turner *et al.,* (1995) also observed that trypanosome stocks that grow faster have higher parasitaemia and greater virulence.

Active surveillance programmes taking place to control sleeping sickness epidemics have reduced disease out brakes in Busia than in Busoga. Therefore, Busia isolates (KETRI 3685 and KETRI 3440) were sensitive to all the suramin and melB dosages while the Busoga isolate EATRO 1886 was resistant to suramin at 2.5mg/kg and sensitive to others. This suggests that in Busoga during eruptions of frequent epidemics, parasites encountered frequent drug exposure. Therefore, they tend to adapt and develop resistance towards the drugs. Murray, (1979) in a sensitivity study on *T. b. rhodesiense* concluded that drug resistance reduced the virulence of the parasite to the vertebrate host. This explains the situation of EATRO 1886 in this study which was resistant to Suramin at 2.5mg/kg and emerged to be chronic.

In conclusion this study has shown clear differences in pathogenicity between clones and their respective isolates, Busia isolates and their respective clones were stil sensitive to dosages of the drugs used but some for Busoga were not with EATRO 1886 resistant to suramin at 2.5mg/kg of bodyweight.

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Table 1: Characteristics of the isolates and their respective clones.

Isolates	Clones	Year isolated	Host isolated from	Place of isolation
KETRI 3340	KETRI 3664	1987	Human	Busia
EATRO 1886	KETRI 3305	1971	Human	Busoga
EATRO 2285	KETRI 3380	1976	Human	Busoga
KETRI 3685	KETRI 3800	2000	Human	Busia

Table 2: Experimental set up for every stabilate

Group	Drug	Dose (mg/kg body weight)	No of mice
1	Suramin	2.5	5
2	Suramin	5.0	5
3	Mel B	1.0	5
4	Mel B	20.0	5
5	-	Untreated	10

Table 3: Pathogenicity of different *T. b. rhodesiense* isolates and clones

Isolate number	**Mean pre-patent period (range)**	**Mean survival time (range)**	**Year of isolation and place**
KETRI 3685 (isolate)	7 (5-7)	38 (31-49)	2000 (Busia)
KETRI 3800 (clone)	3 (1-5)	20 (16-36)	
KETRI 3440 (isolate)	4 (3-5)	33 (21-49)	1987(Busia)
KETRI 3664 (clone)	3 (3-5)	35 (10-50)	
EATRO2285 (isolate)	4 (3-8)	47 (7-57)	1976 (Busoga)
KETRI 3380 (clone)	3 (3-6)	21 (7-36)	
EATRO1886 (isolate)	5 (3-10)	44 (25-60)	1971(Busoga)
KETRI 3305 (clone)	6 (3-10)	34 (30-44)	

Table 4; Sensitivity of different *T. b. rhodesiense* isolates and clones to tyrpanocidal drugs

Stabilate number	**Number of mice cured**				**Time of relapse (days)**			
	Mel B 1mg/kg	**Mel B 20mg/kg**	**Suramin 2.5mg/kg**	**Suramin 5mg/kg**	**Mel B 1mg/kg**	**Mel B 20mg/kg**	**Suramin 2.5mg/kg**	**Suramin 5mg/kg**
KETRI 3800	5/5	5/5	5/5	5/5	0	0	0	0
KETRI 3685	5/5	5/5	5/5	5/5	0	0	0	0
KETRI 3440	5/5	5/5	5/5	5/5	0	0	0	0
KETRI 3664	5/5	5/5	5/5	5/5	0	0	0	0
KETRI 3380	5/5	5/5	5/5	5/5	0	0	0	0
EATRO2285	5/5	5/5	5/5	5/5	0	0	0	0
KETRI 3305	5/5	5/5	5/5	5/5	0	0	0	0
EATRO1886	5/5	5/5	0/5	4/5	0	0	17	17

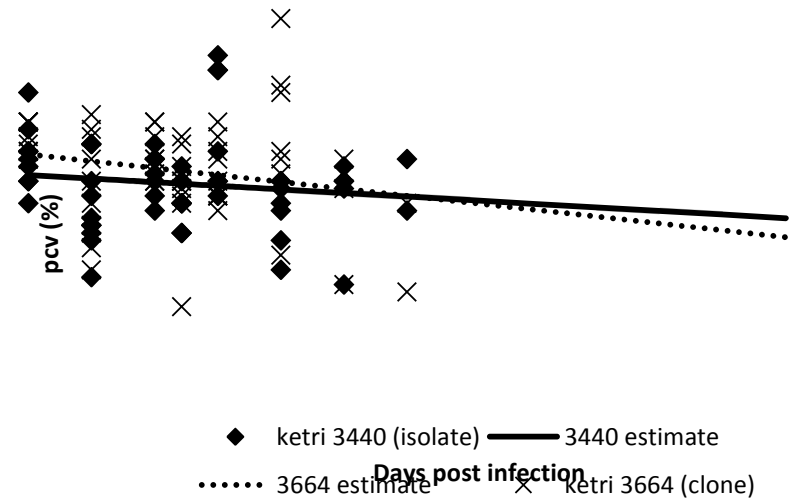

Fig. 3.1 : The fitted regression line for KETRI 3440 (isolate) and KETRI 3664 (clone

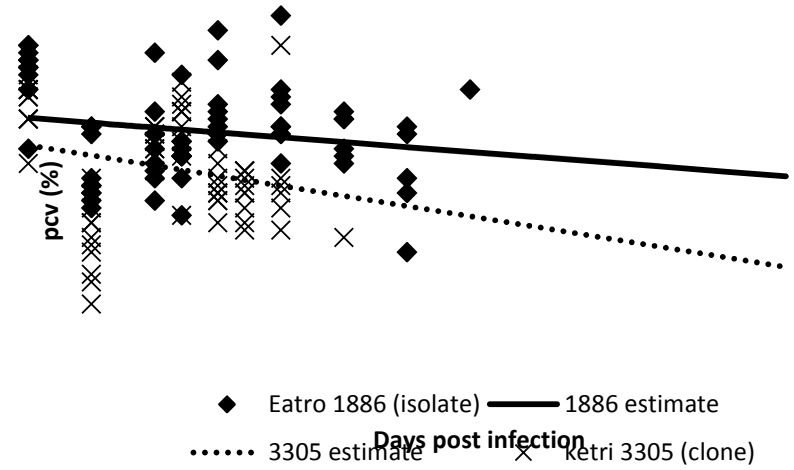

Fig. 3.2: Fitted regression line of EATRO1886 (isolate) and KETRI 3305

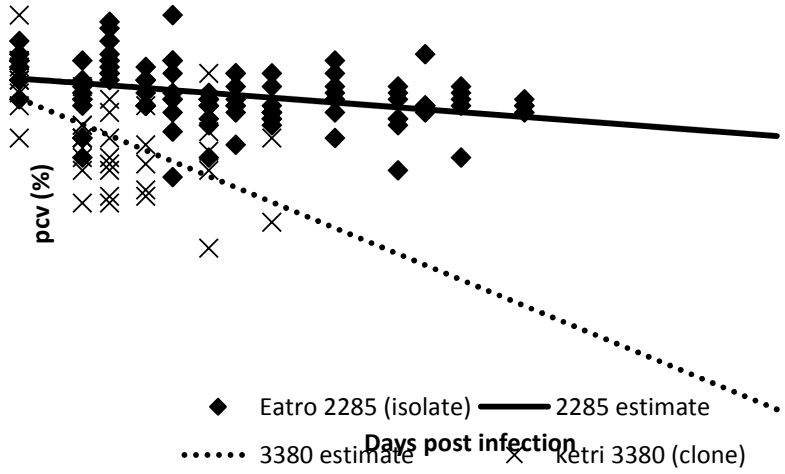

Fig. 3.3: Fitted regression line of EATRO 2285 and KETRI 3380

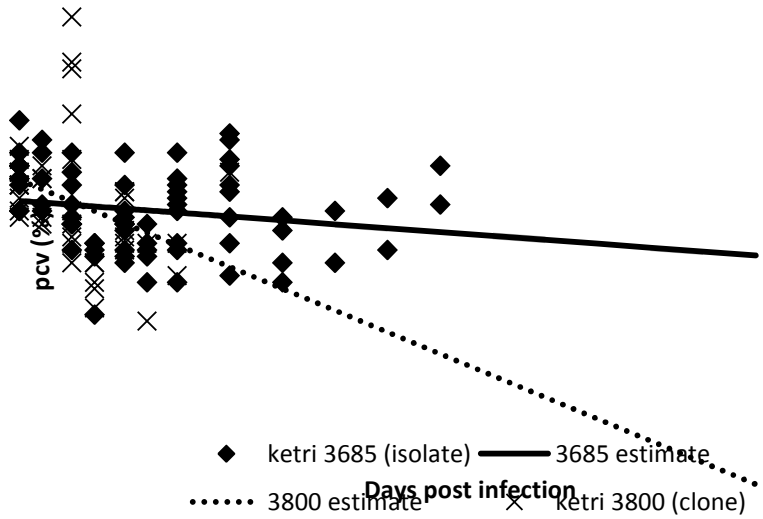

Fig. 3.4: Fitted regression line of KETRI 3685 and KETRI 3800

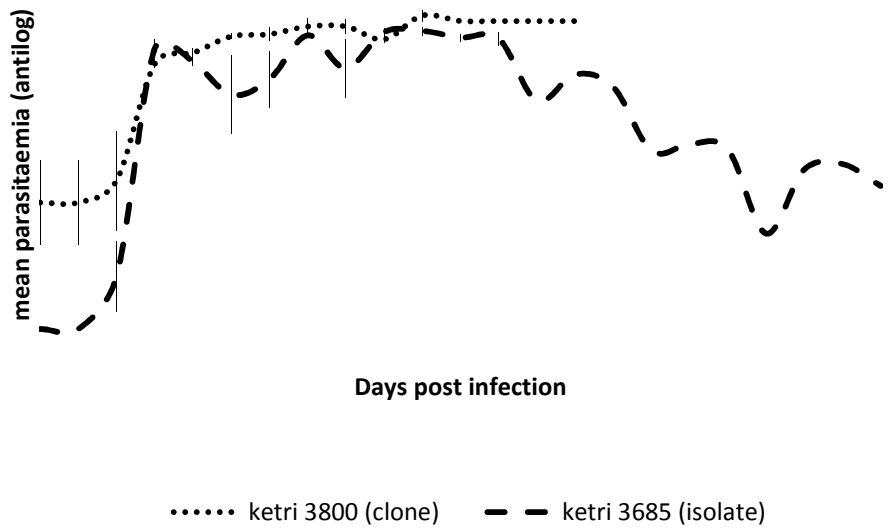

Fig. 3.5: Mean parasitaemia of KETRI 3685 (isolate) and KETRI 3800 (clone) against DPI