Susceptibility of *Simulium damnosum* complex larvae to temephos in the Tukuyu onchocerciasis focus, southwest Tanzania

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Abstract: Tukuyu onchocerciasis focus was earmarked for vector control using insecticide against larval stages**.** Susceptibility tests of mature larvae of *Simulium damnosum* s.l. vectors to temephos insecticide were carried out before and after two years of insecticide treatment of rivers within Tukuyu onchocerciasis focus, south-western Tanzania. The tests were done in 1999/2000 and 2004 using WHO standard methods. Mature larvae were exposed to 9 concentrations of temephos active ingredient, from the weakest 0.00975mg/litre to the strongest of 2.5mg/l. Each test concentration and control was run in duplicates of 25 larvae each, set for three hours in a cool temperature. After incubation, test solution was discarded and larval condition checked. Numbers of larvae in each category were recorded and used to determine mortality rate for each concentration as well as for the LC_{50} and LC_{95}. A total of 1,666 larvae were tested, 942 during the pre- and 724 post-treatment. Results showed that both pre and post-treatment samples were susceptible, attaining 100% mortality at the diagnostic dose of 1.25mg/l, and LC_{50} between 0.129-0.34mg/l pre - and 0.144-0.211 mg/l (95% CI, P<0.05) post- treatment. These values fall within the standard diagnostic dose of ≤0.4mg/l for susceptible *S. damnosum* s.l populations. It was concluded that the endemic *S. damnosum* population was susceptible to temephos before and after two years of intermittent field application. Temephos was thus recommended for continued use in onchocerciasis vector control in the Tukuyu focus, to complement Community Directed Treatment with Ivermectin, but close monitoring of vector susceptibility should be done.

Key words: susceptibility, *Simulium damnosum,* control, temephos, Tanzania

Introduction

Onchocerciasis is a disease caused by a filarial nematode *Onchocerca volvulus*. Adult worms dwell in subcutaneous nodule, from where fertilized females produce millions of microfilariae (mf) which live and freely migrate in the intercellular spaces of the skin tissues, where they cause a wide spectrum of signs and symptoms of skin disease (onchodermatitis). The skin diseases is usually characterised by an acute itching and pruritis which may progress to chronic skin changes leading to loss of elasticity, skin thickening (elephant or lizard skin) and premature ageing. The chronic scratching may lead to pigment changes as in "leopard skin" or depigmentation and dispigmentation. Other manifestations associated with onchocerciasis are hanging groins, hernia and elephantiasis. When the microfilariea invade the eye tissues, they cause damage to different parts of the eye including the cornea, conjunctiva, anterior and posterior eye segments, which may lead to a range of eye defects from loss of visual acuity to irreversible blindness (Buck, 1974). Due to these life long chronic manifestations, the disease is a serious socioeconomic impediment, as it occurs in fertile river valleys most suited for agricultural exploitation.

Onchocerciasis is a tropical disease, endemic in 28 countries in Africa, 6 in Latin America and in the Yemen. An estimated 18 million people are infected, over 99% of these live in Africa, where the disease is responsible for the loss of 1 million DALYs annually (WHO, 2002). The disease endemic zone stretches across the middle belt of Africa, from Senegal in the west to Ethiopia in the East, on its northern limits and in the south, from Angola to southern Malawi. Tanzania lies within these limits, where the disease epidemiology shows a focal distribution due to the specialized vector ecology.

In 1993, it was estimated that about 650,000 people were infected with *O. volvulus* (WHO, 1995) in different foci of Tanzania, the estimate which has since been updated to over a million since the launching of the national onchocerciasis control programme, which was preceded by country wide rapid epidemiological mapping activities (Ministry of Health Unpublished Document, 1997). Of the historically well known foci, the disease prevalence was reported as high in the Bwakira area of southern Uluguru Mountains (63.6%), Mahenge (58.6%), and Ruvuma (31.9%); and low in Amani (22.4%) and Tukuyu (22.8%) (Mwaiko *et al.,* 1990). Previously, a mean onchocerciasis infection rates of 28.7% (range= 2.9%-62.8.4%) had been reported by Pederesen & Kolstrup (1986).

Onchocerciasis is transmitted by a female black fly of the *Simulium* species. The intensity of transmission and level of disease endemicity depends

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on the various vector characteristics, from a suitable ecology and population dynamics of the vector as well as vectorial capacity and human socio-economic activities. The vector black flies are active during daytime and outdoors, so they make contact with their hosts in these circumstances.

Until 1986, the disease control campaigns mainly focused on vector control, due to lack of safe drugs for human use. The current interventions include community directed treatment with ivermectin (CDTI) in the 19 endemic countries including Tanzania. In addition, in three of these, focal vector control / elimination projects were recommended. One such a project was earmarked for the Tukuyu focus, to compliment mass ivermectin treatment, and eliminate the parasite reservoir within a shorter duration than it would otherwise take.

Vector control using temephos was the mainstay of the Onchocerciasis Control Programme in West Africa from 1974 to 2002 (Boatin *et al*., 1997; Borsboom *et al,* 2003). The insecticide was considered the most environmentally friendly compound whose end products were biodegradable and left no lasting residues in the aquatic environment. The "*Simulium formulation*" was 20% emulsifiable concentrate (20% EC), which was sprayed from the air by planes over flying target river stretches, to achieve large coverage in a short time, on weekly basis. The aerial spraying was planned to kill vector larval stages before emerging into adults, thus preventing parasite transmission from one person to another for as long as vector control was effective (Davies, 1994; Walsh"*et al.,* 1981).

The onchocerciasis vector in the Tukuyu focus was first identified as the Kiwira form of the *S. damnosum* complex (Maegga, 1992; Maegga & Cupp, 1993, 1994), which was later confirmed as *Simulium thyolense* (Mustapha *et al.*, 2005). The most predominant cytospecies was previously identified as a vector in the Thyolo highlands onchocerciasis focus of Southern Malawi (Vajime *et al.,* 2000). No vector control campaign against onchocerciasis had ever been done in Tanzania before the Tukuyu focus vector elimination trials of 2003 and 2005. However, a possibility for the existence of vector innate tolerance and /or exposure to agricultural insecticides or other ecological determinants could not be ruled out, thus measuring levels of susceptibility of *S. damnosum* s.l. larvae to temephos was an essential prerequisite to making the final decision whether to use the particular insecticide or not, and once adopted, for how long it may continue to be used. The study was therefore carried out to determine the susceptibility of untreated populations of *S. damnosum* s.l. larvae to temephos insecticide prior to vector control campaign, and to monitor the susceptibility levels during the course of the campaign for evidence of insecticide tolerance or resistance evolution in the meantime.

Materials and Methods

Study area

Tukuyu Onchocerciasis focus covers Rungwe, Kyela and Ileje districts in south-western Tanzania. The focus is located between $9^0$05'S to $9^0$45'S and $33^0$20'E to $34^0$20 2E, in an area of slightly over 3,000km^2. In 2004, about 98,641 people in the Tukuyu focus were estimated to be infected and approximately 300,000 were at risk of contracting the infection (Ministry of Health, 2004 unpubl.).

Larval sampling sites where susceptibility test specimen was collected from, were selected from river stretches known to have dense *S. damnosum* s.l. larval populations during the dry season. These included Rivers Kiwira (at Lema), Mbaka (at Kambasegela) and Lufilyo (at Tapio Bridge). The same points were also used for adult fly catching to monitor transmission of onchocerciasis in the area, as first described by Pedersen & Maegga (1985). Therefore it was highly probable that the *S. damnosum* s.l. sampled for susceptibility testing belongs to the local vector species, *S. thyolense.*

Study design

The tests were based on established method of WHO (1981), originally field tried by Mouchet *et al.* (1977). Five *in-situ* susceptibility tests were conducted of which three tests were for untreated population as baseline data in year 1999/ 2000. The other three tests were conducted in 2004 as monitoring for the population under treatment. Larvae were collected from breeding sites while still attached to their supports in icebox and transported to the sub-bases. Larvae were picked off the substrates, and lifted with soft forceps and placed in the test enamel bowls containing about 50ml test solution. Only 6th and 7th instars (mature larvae) were chosen for the test. At least two replicas were set up for each concentration. *S. damnosum* s.l. larval instars were morphologically identified by standard keys as described by Freeman & De Meillon (1953) and Crosskey (1969). Sight identification of mature larvae of sixth and seventh instars was done using a hand lens to check for cuticular setae extending forward to the proleg, and pairs of prominent dorsal – lateral abdominal tubercles or bumps. Confirmation of larval identity after the

test was done under a low power dissection microscope. Specific characteristics checked for the *S. damnosum* complex were the presence and distribution of the cuticular setation and larval development instar criteria of the presence and size of thoracic black spots.

Susceptibility tests

Temephos was mixed with test water in 500ml bottles at 19-22^{0}C. The solutions of temephos were prepared according to protocol of testing the susceptibility of larvae of *S. damnosum* complex (WHO, 1981, 1983) in ascending series of concentrations of 0.00975, 0.0195, 0.039, 0.078, 0.156, 0.3125, 0.625, 1.25, and 2.5mg/l. The solutions were thoroughly mixed by swirling. The water in which larvae were immersed in the test bowls was carefully tipped out without dislodging the larvae and insecticide solution carefully added. Fifteen minutes intervals were left between different concentrations to allow for harvesting time and scoring of each set after the 3 hours of incubation. The control bowls had a solution of a measured amount of absolute ethanol used in diluting temephos mixed with water. Each test series had two control bowls for comparison. All bowls were appropriately labelled using felt-tipped marker and placed in isothermic cabinet for temperature mitigation.

At the end of the 3 hour incubation period, larvae were separated into three groups of live, moribund and dead. Live larvae were characterized by contracting quickly into "U" shape with the head touching the posterior end when the edge of the bowl was rapped or when larvae touched with forceps. Moribund larvae were identified by their sluggish movement when touched where as dead larvae were recognized by being immobile and not sensitive when touched by forceps. At every concentration, removal of larvae followed a trend live, moribund and finally dead. Each category at each concentration was preserved in 80% alcohol in separate tubes. Final sorting and counting of live, moribund and dead larvae were done in the laboratory with the aid of dissection microscope. Only the mature larvae of 6th and 7th instars were included in test counts, while those that were too young or those classified as pharate pupae were rejected, the separation was done based on morphological criteria for larvae inclusion in these test. At times larvae of species other than *S. damnosum s.l.* , especially *S. hargreavesi*, were also included as often it was difficult to separate them by sight alone. These were also discarded once confirmed by microscopy.

Data analysis

Percentage mortality of larvae was calculated from moribund and dead larvae for each set of tests. In tests replicas where control mortality exceeded 5%, but was less than 20%, the test mortalities were corrected by Abbott's formula shown below:

$$\% \text{ corrected mortality} = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100\% - \% \text{ control mortality.}} \times 100$$

When the control mortality exceeded 20% the whole test was discarded and the exercise repeated after correction of any presumed errors. PoloPlus software package (version 1.0, LeOra Software, 2002-2006) was used to determine LC_{50} from the dosage mortality curve. LC_{50} up to 0.4mg/l is accepted value for susceptible population whereas LC_{50} from 0.4mg/l to 1.0mg/l indicates intermediate susceptibility, and LC_{50} above 1.0mg/l is a sign of resistance.

Results

A total of 1,666 larvae of *S. damnosum* complex, (assumed to belong to *S. thyolense*), of 6th and 7th instars were tested. Out of these, 942 were a sample before treatment and 724 after treatment. Samples before treatment were divided as follows: 405 from Mbaka, 158 from Lufilyo and 379 from Kiwira. For the follow-up samples, the breakdown was: 422 were from Mbaka, 302 larvae were from Lufilyo. During the latter study, there was no enough *S. damnosum s.l.* of the right size on the Kiwira river site.

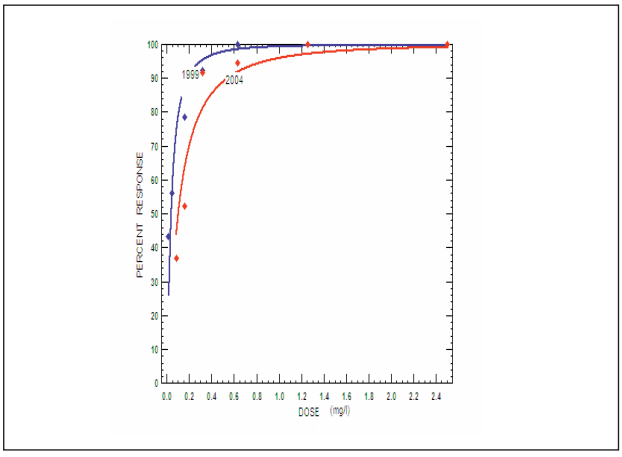

Figure 1: susceptibility to temephos: Percent mortality of mature larvae of *S. damnosum* s.l. at different concentrations in the Mbaka River, Tukuyu focus

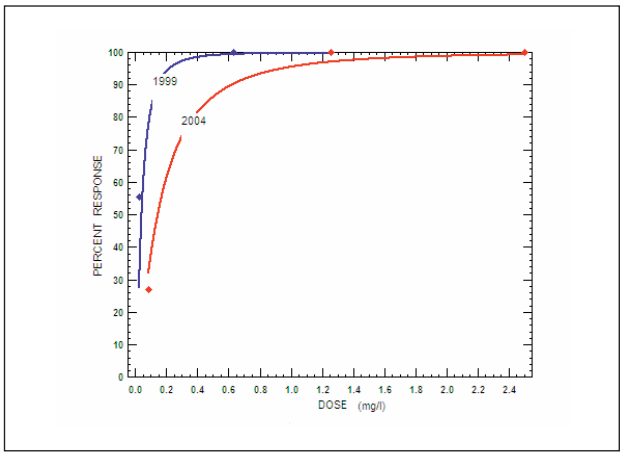

Figure 2: Percent mortality of mature larvae of *S. damnosum* s.l. at different concentrations in the Lufilyo River

In the pre-treatment samples, mortality rate of 100% was attained with 1.25mg/l, which is the standard diagnostic dose for *S. damnosum s.l.* vector species. The LC_{50} was observed to range from 0.129 - 0.34mg/l (95% CI, $P<0.05$), this too was within the standard dose of ≤0.4mg/l for a susceptible population. After two years of intermittent treatment of the area with temephos, larval mortality rate of 100% was attained with the diagnostic dose limit of 1.25mg/l, as for the pre-treatment population. The post-treatment LC_{50} was 0.144-0.211 mg/l (95% CI, $P<0.05$), which was within the standard dose of ≤0.4mg/l for a susceptible population, as in the pre-treatment case. The mean LC_{50} was highest in Kiwira (0.211mg/l), followed by Lufilyo (0.165mg/l) and Mbaka (0.079mg/l) (Figures 1, 2 and 3).

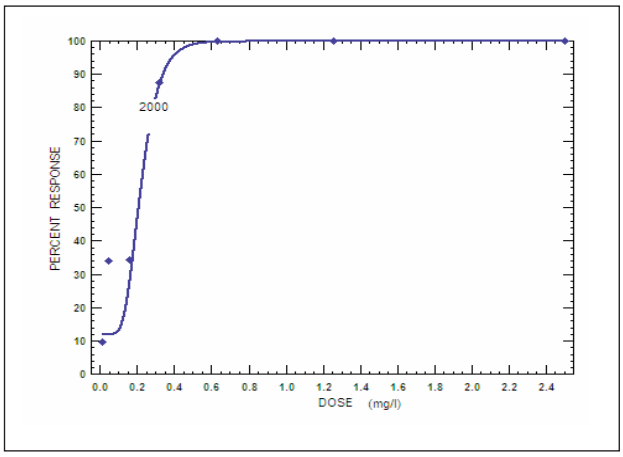

Figure 3: Percent mortality of mature larvae of *S. damnosum* s.l. at different concentrations in the Kiwira River

Discussion

Tukuyu area has a history of agricultural use of organophosphate pesticides, although concrete documentation was hard to find. Thus there was genuine concern of some of these pesticides or their residues trickling into the drainage system, such as springs, streams and eventually into the river systems which form breeding habitats of the *Simulium* species, some of which are important vectors of human onchocerciasis. The persistent presence of such insecticides in the normal ecological habitat could potentially induce insecticide tolerance to both target and non-target aquatic fauna. For this reason, it was an essential pre-requisite to determine levels of susceptibility of *S. damnosum* s.l. vectors to this new insecticide, before making final decision to use it as the main control tool, otherwise, the decision could prove counter productive if the insecticide was tolerated by the vector species. However, from the results of the pre- and post-treatment susceptibility tests, it was observed that *S. damnosum* s.l larval populations in the local rivers were very susceptible to the insecticide, and thus, could be used for its control. The indicators of a susceptible population used in these observations were: the lethal concentration range of 0.129 - 0.34mg/l for 50% mortality of the vector population before treatment was within the standard dosage of (≤0.4mg/l), as well as that of the post-treatment population. From the experiences of the Onchocerciasis Control Programme (OCP) areas, mature larvae of *S. damnosum* s.l. were regarded to be resistant to temephos when they survive concentrations higher than 1.0 mg/l (Davies, 1994).

In the current study, 100% mortality was attained at a relatively low concentration of 0.65 mg/l), a clear demonstration of the population's susceptibility to temephos 20% EC. From the first set of results of the susceptibility tests conducted on Mbaka, Lufilyo and Kiwira rivers within the focus, the decision was made to organise insecticide treatment campaigns against the local vector, using temephos 20%EC. Then a relatively large scale field vector control feasibility study was conducted for six weeks, from late December 2001 to late January 2002 (data shown elsewhere). After achieving a successful outcome of the large scale feasibility study, the second exercise, which was the first actual vector control campaign was done for 17 weeks in the main Tukuyu focus rivers (20 weeks in Lumbira on the focal fringes), in 2003. In both occasions, vector population was decimated, and the adult biting catches were reduced to undetectable low levels on the three main rivers of

the Tukuyu focus. However, as insecticide treatment was stopped before completed vector elimination was attained, vector breeding slowly begun, and adult human biting females reappeared after several months (data not shown). The post- treatment susceptibility test was therefore done on the vector population that was found following the first actual control campaign. Again, this population was found to be susceptible to temephos insecticide, as shown by the results. Subsequent to these findings, a second large scale insecticide treatment campaign was executed, for 11 weeks of insecticide spraying into rivers to clear Simulium vector larvae, while simultaneously carrying out rigorous monitoring of the vector breeding and biting activities.

From the experience in West African OCP programme, the first evidence of *S. damnosum* s.l. resistance development to temephos was reported approximately five years after continuous intensive application in the upstream parts (WHO, 1995), while the resistant population was found in downstream stretches. That implies that a diluted concentration of the product was reaching downstream populations for a relatively long time before resistance was detectable. In the current study, the insecticide was not continuously used throughout the two to three years' of treatment, but intermittently, for some weeks and then stopped. The re-colonization of breeding sites could have occurred through residual population left during treatment operations or new unexposed populations migrating from nearby areas. The population's duration of exposure to insecticide pressure was probably not long enough for clear resistance development. On the other hand, the intermittent insecticide treatment approach was a useful strategy in delaying resistance development. For instance, in the vector control campaigns in parts of the OCP, this strategy was used in order to ease the intensity of insecticide selection pressure for resistance development, and even after 8 years of intermittent treatment on the Volta River at Sencchi rapids, the local *S. damnosum* s.l. was found to be still susceptible to temephos (Adiamah, 1986). Thus intermittent insecticide treatment in onchocerciasis vector control could also offer a viable alternative strategy where insecticide resistance development is a real threat. In spite of the satisfactory susceptibility levels observed, it is important to note that different genetic populations of the same *S. damnosum* complex show different paces of resistance development with the same insecticide exposure duration (Davies, 1994). This is based on the inherent genetic variability of the different cytotypes, as they are endowed with natural flexibility to withstand or adapt to different ecological niches in their natural habitats. In addition, in many parts of Africa, including Tanzania, environmental pollution from products used in agriculture, livestock and mining industries cannot be ruled out. Since in most cases, no records are available, extreme vigilance is absolutely essential. For instance, in this study, among the three rivers where larvae were sampled, Kiwira river population showed higher mean LC_{50} of 0.211mg/l compared to Mbaka (0.079mg/l) and Lufilyo (0.165mg/l), for the untreated population. The reason for this phenomenon was not immediately clear. However, the presence of a coal mining factory nearby the sampling area on the Kiwira river bank may have exposed its population to some chemical products emanating from the factory since its opening over 10 years ago. Whatever the reason for these differences, it was apparent that the Kiwira population showed higher tolerance to temephos than Lufilyo or Mbaka populations. Furthermore, it was also observed that, while the post-treatment susceptibility tests showed a normally susceptible population, the dose— mortality curve slope was slightly less steep, tending towards greater survival at concentrations lethal for the pre-treatment population. Further investigations are therefore recommended to follow-up this phenomenon very closely.

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Characterisation of the *Trypanosoma brucei rhodesiense* isolates from Tanzania using serum resistance associated gene as molecular marker

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Abstract: Serum resistance associated (SRA) gene has been found to confer resistance to the innate trypanolytic factor (TLF) found in normal human serum; thus allowing *Trypanosoma brucei brucei* to survive exposure to normal human serum. This study was carried out to examine the presence of SRA gene and identify the origin of *T. b. rhodesiense* isolates from three districts in Tanzania, namely Kibondo, Kasulu and Urambo. Twenty-six *T. b. rhodesiense* isolates and two references *T. b. rhodesiense* isolates from Kenya were examined for SRA gene using simple Polymerase Chain Reaction technique. The gene was found to be present in all 26 *T. b. rhodesiense* isolates including the two references isolates from Kenya. The SRA gene was confirmed to be specific to *T. b. rhodesiense* since it could not be amplified from all other *Trypanozoon* including *T. b. gambiense;* and gave an amplified fragment of the expected size (3.9kb), confirming that all these isolates were *T. b. rhodesiense* of the northern variant. Although the geographic distributions of *T. b. gambiense* and *T. b. rhodesiense* are clearly localized to west/central Africa and eastern Africa, respectively, natural movement of people and recent influx of large number of refugees into Tanzania from the Democratic Republic of Congo, could have brought *T. b. gambiense* in western Tanzania. The overlap in distribution of both of these pathogenic sub-species could result in erroneous diagnoses since both trypanosome sub-species are morphologically identical, and currently serologic methods have low specificity. Both the susceptible and resistant *T.b. rhodesiense* isolates possessed the SRA gene suggesting that there is no correlation between drug resistance and presence of SRA gene. The use of SRA gene helps to confirm the identity and diversity of some of the isolates resistant to various drugs.

Keywords: *Trypanosoma brucei rhodesiense*, resistance, SRA gene, PCR, Tanzania

Introduction

Innate protective molecules in the blood of primates influence the host range of African trypanosomes. Human blood, unlike the blood of other mammals, has efficient trypanolytic activity, and this needs to be counteracted by these parasites. *Trypanosoma brucei* consists of three sub-species, non-human infective *Trypanosoma brucei brucei* and human infective *T. b. rhodesiense* and *T. b. gambiense* that are indistinguishable by conventional morphological, biochemical and antigenic criteria but differ by their geographical distribution, host specificity (Mehlitz *et al.,* 1982; Noireau *et al.,* 1989; Hide *et al.,* 1994; Gibson, 2001). A sub-fraction of human high-density lipoprotein (HDL) containing apolipoprotein A-I, apolipoprotein L-I, and haptoglobin-related protein is toxic to *T. b. brucei* but not *T. b. rhodesiense* (Pays *et al.,* 2006).

This question of sub-speciation has been resolved in the past, by inoculating human volunteers with trypanosomes isolated from other animals (Heisch *et al.,* 1958; Onyango *et al.,* 1966), or more acceptably by *in vitro* tests involving incubation of trypanosomes with human blood in the blood incubation test (BIIT) (Rickman & Robson, 1970) or with serum in human serum resistance test (HSRT) (Brun & Jenni, 1987). Biochemical and molecular characterization such as isoenzyme electrophoresis, restriction fragment length polymorphisms using ribosomal DNAs (RFLP) have shown significant differences between the two subspecies, but have not defined clearly the criteria for identifying the human-infective sub-species isolated from animal reservoirs or vectors. However, these methods are time consuming, laborious and inappropriate for the field situation (Gibson, 1989; Hide *et al.,* 1990, 1994, 1998). Therefore, the search for techniques to distinguish *T. b. rhodesiense* from *T. b. brucei* and hence its human infectivity is important.

T. b. brucei causes nagana in cattle but is not pathogenic in humans because this sub-species is lyzed by high-density lipoproteins (HDL) present in human serum. It is thought that *T. b. rhodesiense* evolved from a *T. b. brucei*-like ancestor and expresses a defence protein that ablates the anti-trypanosomal activity of human HDL. The ability of *T. b. rhodesiense* and *T. b. gambiense* to be resistant to normal human serum (NHS) enabled them to parasitize humans and cause sleeping sickness. The mechanism of resistance to NHS is still a subject for debate but previously it was believed to be due to defect in the uptake of HDL factor (Hager & Hajduk, 1997).

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This search has been nearly concluded by the discovery of the mechanism of human serum resistance in *T. b. rhodesiense.* A single gene, known as serum resistance associated (SRA) gene has been found to confer resistance to the innate trypanolytic factor (TLF) found in normal human serum. This gene allows *T.b brucei* to survive the exposure to normal human serum (Xong *et al.,* 1998). In long, slender bloodstream forms of *T. b. rhodesiense*, the expression of SRA allows neutralization of APOL1 in the lysosome. This mechanism of resistance considerably differs from the previously proposed mechanism, the selective inhibition of endocytosis of the trypanolytic factor.

The SRA gene was isolated for the first time from *T. b. rhodesiense* isolate from Uganda (De Geef *et al.*, 1989). The SRA gene is transcribed from one of the multiple telomeric loci where variant surface glycoprotein (VSG) genes are expressed (VSG expression sites). This expression site is selected when trypanosomes are grown in presence of NHS (Van Xong *et al*., 1998) The product of SRA is a atypical VSG of shorter than average length being 410 amino acids instead of approximately 490 (Vanhamme *et al.*, 2003).

The human SRA gene has been found in all *T. b. rhodesiense* isolates examined from sleeping sickness foci throughout East Africa, but not in *T. b. brucei* or any other trypanosomes of subgenus *Trypanozoon*, including *T. b. gambiense* (De Greef *et al.,* 1992; Welburn *et al.,* 2001; Radwanska *et al.,* 2002; Gibson *et al.,* 2002). This suggests that *T. b. gambiense* resists lysis through a different mechanism. Indeed *T. b. gambiense* and *T. b. rhodesiense* appear to differ in their mechanism of resistance to normal human serum. In contrast to *T. b. brucei*, the subspecies *T. b. gambiense* and *T. b. rhodesiense* escape the trypanolytic activity of human serum and cause sleeping sickness pathology. *T. b. gambiense* is permanently resistant to human serum whereas *T. b. rhodesiense* loses resistance after being isolated from humans and transferred to other animals (Hawking, 1977).

Recent advances in this field of research include a breakthrough in the diagnosis of sleeping sickness, for which the presence of SRA has proved to be a reliable marker of infection with *T. b. rhodesiense* (Gibson 2005; Gibson *et al,* 2002; Welburn *et al.,* 2001). This marker can also be used to distinguish *T. b. rhodesiense* from *T. b. gambiense*.

In this study, the SRA characterization was undertaken because the study area in western Tanzania is home to thousands of refugees from the highly *T. b. gambiense* endemic country, the Democratic Republic of Congo. It was thought that natural movement of people and influx of large number of refugees could have brought *T. b. gambiense* into western Tanzania, and also some of the isolates, both drug resistant and sensitive, identified probably could be cases of T. *b. gambiense* and not *T. rhodesiense.* The SRA marker was also used to characterize geographical origin of the isolates because this has migration implications that influence spread of drug resistant strains in the region.

Materials and Methods

Study areas

The study area included Kibondo, Kasulu and Urambo Districts in western Tanzania. The study area has been described in detail by Malele *et al*. (2006). Purposeful sampling method was used to select Kibondo, Kasulu and Urambo, because of high human African trypanosomiasis reporting cases for the past five years (Ministry of Health, unpubl.).

Isolation of trypanosomes

During the survey, people suspected to be infected with trypanosomes based on clinical observations were examined by blood smear and haematocrit centrifugation technique (HCT) in order to confirm the infection. Blood samples (2ml) were collected from confirmed sleeping sickness cases by venipuncture under the supervision of a medical doctor. Then, the patients were referred to hospital for treatment according to the stage of their illness. Each blood sample collected was divided into two portions, which were cryopreserved in liquid nitrogen. One portion was used for propagation of the isolates to the mice and the other was kept for future references.

DNA extraction from T. b. rhodesiense isolates

Genomic DNA was extracted using a commercial kit (Puregene DNA isolation kit D-7000A, Gentra Systems, Minneapolis, USA) following the manufacturer‘s instructions with minor modifications. A total of 500ml of blood was mixed with 1500ml of RBC lysis solution and incubated at room temperature for 5 minutes. The mixture was then span at 13000g for 2 minutes and the supernatant discarded. The pellet was resuspended in about 50ml of residual fluid, which was mixed with 250ml cell lysis solution containing proteinase K (100mg/ml), and incubated at 55^{o}C for 1 hour. RNAse (29mg/ml) was then added and the mixture incubated at 37^{o}C for another 45 minutes.

Protein was then isolated by addition of 200ml of protein precipitation solution, and incubated on ice for 5 minutes, followed by micro-centrifugation for 5 minutes at maximum speed. From that supernatant, DNA was precipitated by addition of 600 ml isopropanol, and the pellet was washed with 70% ethanol. The dry pellet was finally dissolved in 20ml of the DNA hydration solution included in the kit and allowed to rehydrate at 65°C for 1 hour.

PCR analysis for* T. brucei *subgroup

Primers used to amplify species specific DNA targets for *T. brucei* subgroup from isolates were TBR 1 and 2 with sequence TBR 1 5'-CGA ATG AAT AAT AAA CAA TGC GCA GT-3' and TBR2 5'-AGA ACC ATT TAT TAG CTT TGT TGC-3' (Artama *et al.,* 1992). The concentration of the primers was 0.4μM. Standard PCR amplifications were carried out in 25μl reactions mixtures containing the final concentrations, 10 mM TrisHCL pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μm of each of the 4 deoxynucleoside triphosphates and 1 unit of *RED Taq* DNA polymerase (Sigma). The amplification conditions were (30 cycles) 94°C for 30 sec, 60°C for 60 sec and 72°C for 30 sec. PCR products were separated by electrophoresis in 1.5% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide and visualized under ultraviolet light.

SRA amplification from genomic DNA

PCR primers for SRA amplification were based on the DNA sequence of the SRA gene and its homologues to Kenyan *T. b. rhodesiense* isolates (EMBL accession number AF097331). Primers B537 (forward) (5'-CCA TGG CCT TTG ACG AAG AGC CCG-3') and B538 (reverse) (5'-CTC GAG TTT GCT TTT CTG TAT TTT TCC T) at 2'μM were complimentary to the 5' and 3' ends of the published SRA gene (accession no. AF097331). These primers were used to screen all trypanosome genomic DNA from 28 isolates for presence of SRA fragment by PCR (Welburn *et al.,* 2001). The amplification condition was 30 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 90 sec. Reaction volumes (25μl) which contained 1 U HotStarTaq DNA polymerase (Qiagen) supplemented with 1 U *Pfu* polymerase (Promega) and 4 mmol/L $MgCl_2$ were used. In each experiment positive controls for *T. b. brucei, T. b. gambiense* and *T b. rhodesiense* were used.

Screening for northern and southern SRA gene variants

The primers were used to screen trypanosome genomic DNA in order to identify the category (northern or southern) of SRA gene of the isolates; forward SRA H: (5'-GTACCTTGGCGCGCTCC CTGG-3') and reverse SRAJ :(5'-GTA CCT TGGCGCGCT CGCGCTG-3') (Gibson *et al.,* 2002). PCR conditions for this amplification were denaturations at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 60 sec.

Ethics declaration

This study was approved by Medical Research Coordination Committee of the National Institute for Medical Research, Tanzania. The free and informed consent of all patients who participated in this study or their legal guardians was obtained before commencement of the study

Results

Confirmation of* T. brucei *subgroup

All isolates were first confirmed as *T. brucei* subgroup by PCR analysis (Figure 1) using *Trypanozoon* primers TBR1and TBR2 before screening them for the presence of SRA gene. Since the expected band of size of 177bp was present in all, isolates screened were confirmed to belong to *T. brucei* subgroup.

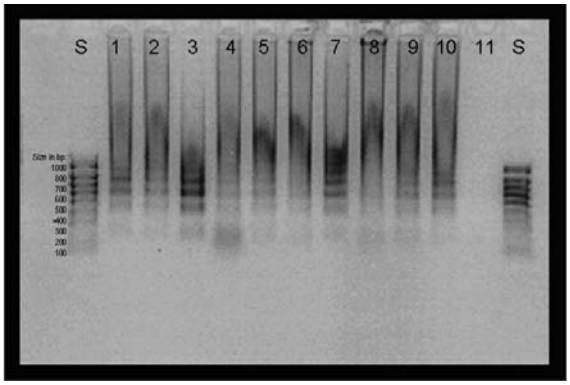

Figure 1: PCR for the confirmation of *T. brucei* subgroup of the isolates studied. Representative samples 1-10 were screened by PCR using Trypanozoon primers, TBR1 and TBR2. Lane 11 corresponds to a negative control. Lane S contains the 100 bp marker (Bioline-UK). Lane 1-10 represents isolates TMRS 3(9), TMRS 10(6), TMRS 11(2), TMRS 12 (2), TMRS 13 (2), TMRS 4 (1), TMRS 1 (13), TMRS 2 (11), KETRI 1989 and KETRI 2653, respectively. The presence of expected PCR product s of 177bp or multiples thereof confirms the isolates as *T. brucei* subgroup

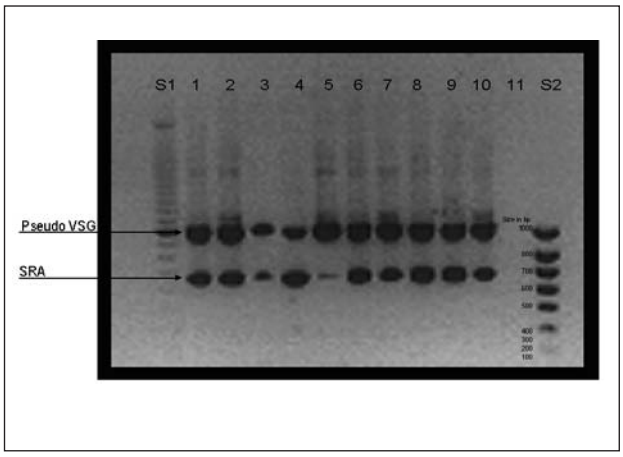

Figure 2: PCR for the identification of SRA gene among the isolates studied. Isolates were screened by PCR using primers B537 and B538 to amplify SRA gene. Lane S1 contains Hyperladder 1. Only representative isolates are shown in Lane 1-10, namely**,** TMRS 3(9), TMRS 10(6), TMRS 11(2), TMRS 12(4), TMRS 13(2), TMRS 4(1), TMRS 1(13), TMRS 2(11), KETRI 1989 and KETRI 2653 respectively. Lane 11 contains negative control and Lane S2 contains 1000bp marker. The presence of expected PCR product of 670bp or multiples thereof confirms the presence of SRA gene.

Amplification of SRA gene

The SRA fragment of approximately 670bp (Figure 2) was amplified from all 26 *T. b. rhodesiense* isolates including 2 reference isolates from Kenya. Thus the SRA gene was present in both drug susceptible and drug-resistant *T. b. rhodesiense* isolates (Table 1).

Screening for the northern and southern SRA gene variants

To determine whether they belong to northern or southern origin these isolates were screened by PCR using SRA primers (SRA H and SRA J). A prominent band of 3.9kb, was amplified from all *T. b. rhodesiense* isolates from Tanzania except two isolates probably due to technical error. The 3.9kb band was also amplified from reference strains KETRI 1989 and KETRI 2356 from Kenya. No band of 3.5kb, which is a marker for southern variant, was amplified from these isolates. All the 24 isolates from Tanzania had the northern variant of the SRA gene (Table 1). The SRA gene was specific for *T. b. rhodesiense* and could not be amplified from *T .b. gambiense* and *T. b. brucei*.

Table 1: Human SRA gene amplification from T. b. rhodesiense genomic DNA

Isolate	Origin	Year	Drug sensitivity status*	SRA**	Category***
TMRS 10 (6)	Kasulu	1999	R IS(1mg)	+	N
TMRS 11 (2)	Kasulu	2000	R M (5mg); D (14mg)	+	BA
TMRS 12 (4)	Kasulu	1999	R M (5 and 10mg); D(14 and28mg)	+	N
TMRS 13 (2)	Kasulu	1999	S	+	N
TMRS 15 (6)	Kibondo	1999	S	+	N
TMRS 7 (2)	Kibondo	1999	S	+	N
TMRS 3 (2)	Kibondo	1999	S	+	N
TMRS 3 (3)	Kibondo	1999	S	+	N
TMRS 3 (11)	Kibondo	2002	R M (5mg)	+	N
TMRS 3 (6)	Kibondo	2002	S	+	N
TMRS 4 (1)	Urambo	2002	R SU (5mg)	+	N
TMRS 2 (2)	Urambo	2002	S	+	N
TMRS 1 (13)	Urambo	2002	R IS (1mg)	+	N
TMRS 5 (1)	Tabora	2000	S	+	N
TMRS 3 (7)	Kibondo	2000	S	+	N
TMRS 9 (5)	Kasulu	2002	S	+	N
TMRS 2(11)	Urambo	2002	R SU (5mg); IS (1mg)	+	N
TMRS 8 (13)	Kasulu	2001	S	+	N
TMRS 3 (9)	Kibondo	2000	S	+	BA
TMRS 10 (3)	Kasulu	2000	S	+	N
TMRS 10 (4)	Kasulu	2000	S	+	N
TMRS 11 (3)	Kasulu	2000	S	+	N
TMRS 12 (2)	Kasulu	2000	S	+	N
TMRS 1 (2)	Urambo	2001	S	+	N
TMRS 3 (12)	Kibondo	2002	S	+	N
TMRS 12 (3)	Kasulu	1999	S	+	N
KETRI 1989	Kenya	1989	R SU and D	+	N
KETRI 2356	Kenya	1997	R M	+	N

*Drug sensitivity status is given as resistant (R) or sensitive (S).
M=Melarsoprol, D= Diminazene, SU= Suramin, IS= Isometamidium; BA= Expected but not amplified
Dosage of drug at which the isolate is resistant is given in brackets.
** The presence of SRA gene is indicated by + signs; *** All isolates were of the Northern (N) variant except two isolates which could not be classified since no band was amplified from them.

Discussion

Several studies have shown that *T. b. rhodesiense* and *T. b. gambiense* are able to infect humans due to their resistance to the cytotoxic action of normal human serum. A subfraction of human high-density lipoprotein (HDL) containing apolipoprotein A-I, apolipoprotein L-I, and haptoglobin-related protein is toxic to *T. b. brucei* but not the human sleeping sickness parasite *T. b. rhodesiense* and *T. b. gambiense* (Faulkner *et al.,* 2006; Pays *et al.,* 2006*).* These two sub-species are largely indistinguishable from *T. b. brucei* except for their resistance to the cytotoxic action of human serum and their ability to infect humans. Therefore, the defining phenotypic trait of human sleeping sickness trypanosomes is their resistance to TLF-mediated lysis. The human serum resistance associated (SRA) gene first isolated from a Ugandan strain of *T. b. rhodesiense* has been shown to be capable by itself of conferring resistance to antitrypanosomal activity of human HDL and the trait of human infectivity on *T.b. brucei* by transfection (Gibson *et al.,* 2001).

To directly investigate this possibility, a study was carried out elsewhere for *in vitro* selection to generate human HDL-resistant *T. b. brucei*. The results showed that conversion of *T. b. brucei* from human HDL sensitive to resistant correlates with changes in the expression of the variant surface glycoprotein (VSG) and abolished uptake of the cytotoxic human HDLs (Faulkner *et al.,* 2006). These findings demonstrate that resistance to human HDLs can be acquired by *T. b. brucei.* Furthermore *T. b. rhodesiense* is likely to have arisen as a clone of *T. b. brucei* that differs mainly or solely by its ability to express SRA on selection in human serum. This gene has also been identified in several other isolates of *T.b. rhodesiense*, but not in *T. b. brucei* or any other trypanosomes of subgenus *Trypanozoon*, including another human infective *T. b. gambiense*. This gene has been found in all *T. b. rhodesiense* isolates examined from sleeping sickness foci in Tanzania confirming that it is found throughout East Africa (Ethiopia, Uganda, Kenya, Rwanda, and Zambia).

The 26 stocks of *T. b. rhodesiense* from patients in 3 districts of Tanzania were examined for the presence of the SRA gene and gave an amplified fragment of the expected size confirming that all these isolates were *T. b. rhodesiense*. Although the geographic distributions of *T. b. gambiense* and *T. b. rhodesiense* are clearly localized to west/central Africa and eastern Africa, respectively, natural movement of people and recent influx of large number of refugees into Tanzania from the Democratic Republic of Congo, could have brought *T. b. gambiense* in western Tanzania. In the Democratic Republic of Congo, the reported prevalence of *T. b. gambiense* sleeping sickness is up to 80% in some foci (Van Nieuwenhove *et al.*, 2001). The overlap in distribution of both of these pathogenic sub-species can result in erroneous diagnoses since both trypanosome sub-species are morphologically identical, and the currently available serologic techniques have low specificity (Radwanska *et al.,* 2002). Nonetheless, since these subspecies show differential drug sensitivities, a correct differential diagnosis between *T. b. gambiense* and *T. b. rhodesiense* is essential for unambiguous diagnosis of drug resistance. Therefore, the development of a simple molecular technique such as the SRA gene-based PCR may be essential for the correct diagnosis of resistance attributable to *T. b. rhodesiense*.

Both the susceptible and resistant *T. b. rhodesiense* isolates possessed the SRA gene suggesting that there is no correlation between drug resistance and presence of SRA gene. The lack of this kind of correlation suggests that these two phenomena arose by independent mechanisms. The drug resistance is most likely to be due to selection by drug pressure (Kibona *et al.,* 2006) and this selection does not affect the SRA gene. It is a valid objective to try to link observed differences in drug sensitivity with genetic markers such as the serum resistance. However, there have been few studies attempting to link drug resistance with other genotypes in East Africa. Matovu *et al.* (1997) carried out *in vitro* screening of the Ugandan *T. b. gambiense* and *T. b. rhodesiense* isolates, identified by human serum resistance (HSR) for susceptibility to melarsoprol, diminazene and isometamidium *in vitro* and found one *T. b rhodesiense* isolate resistant to the tested drugs. In view of that, the potential association between genetic markers and other characteristics such drug resistance or pathogenicity are areas worth to be explored.

The SRA gene cannot be used as sole marker for human infectivity in *T. brucei* sub-species because, it is absent in *T. gambiense* which is human infective. However, since a large variety of wild and domestic animals serve as reservoirs for both subspecies, SRA gene is a useful marker for the identification of trypanosomes of the *T. b. rhodesiense* subspecies that can infect humans (Gibson, 2001). Once the human infectivity of an isolate has been established then SRA gene can be used to distinguish *T. b. rhodesiense* from *T. b. gambiense.*

Although the *SRA* gene is conserved among *T. b. rhodesiense* isolates, there are two major sequence variants, designated northern and southern to reflect

geographical origin (Gibson *et al.,* 2002). This division tallies with previous clinical and molecular characterization studies indicating the existence of northern and southern strains of *T. b. rhodesiense* (Hide & Tilley*,* 2001; Ormerod, 1967; Gibson *et al.,* 1980; Macleod *et al.,* 2000). In this study a prominent band of a 3.9kb was amplified from Tanzanian isolates of *T. b. rhodesiense* genomic DNA. Similar findings have been observed in isolates from Uganda and Kenya (Gibson *et al.* 2002; Gibson & Ferris, 2003), meaning that all these isolates had the northern variant of the SRA gene. Thus the northern variant seems to cover the entire East Africa region including Tanzania, Kenya and Uganda.

Nevertheless, since the SRA gene resembles VSG genes and the extent of genetic evolution of this gene is currently unknown, one cannot exclude the existence of *T. b. rhodesiense* parasites with defective SRA variants or the existence of parasites with a modified SRA gene. Only two SRA genes have been characterized that differ from each other by a few point mutations (De Greef& Hamers, 1994). Moreover, in all cases where this was studied, the SRA gene appears to be a member of a large gene family that contains many pseudo genes; thus, the possibility exists that none of these sequences are functional (De Greef & Hamers, 1994). Recently, Radwanska *et al*. (2002) observed an interesting finding with strain TREU927/4, which is currently used as a reference *T. brucei* strain for genome sequencing. This strain was found to be resistant to lysis by NHS without expression of the SRA gene, even though SRA gene-related sequences were present.

Future studies involving large collections of field isolates are needed to confirm the reliability of the SRA gene PCR in identifying *T. b. rhodesiense* as the causative agent of human trypanosomiasis in different parasite foci.

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