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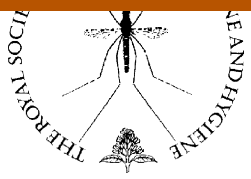

Isolation and propagation of *Trypanosoma brucei gambiense* from sleeping sickness patients in south Sudan

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Summary This study aimed at isolating *Trypanosoma brucei gambiense* from human African trypanosomiasis (HAT) patients from south Sudan. Fifty HAT patients identified during active screening surveys were recruited, most of whom (49/50) were in second-stage disease. Blood and cerebrospinal fluid samples collected from the patients were cryopreserved using Triladyl® as the cryomedium. The samples were stored at −150 °C in liquid nitrogen vapour in a dry shipper. Eighteen patient stabilates could be propagated in immunosuppressed *Mastomys natalensis* and/or SCID mice. Parasitaemia was highest in SCID mice. Further subpassages in *M. natalensis* increased the virulence of the trypanosomes and all 18 isolates recovered from *M. natalensis* or SCID mice became infective to other immunosuppressed mouse breeds. A comparison of immunosuppressed *M. natalensis* and Swiss White, C57/BL and BALB/c mice demonstrated that all rodent breeds were susceptible after the second subpassage and developed a parasitaemia >10^6/ml by Day 5 post infection. The highest parasitaemias were achieved in C57/BL and BALB/c mice. These results indicate that propagation of *T. b. gambiense* isolates after initial isolation in immunosuppressed *M. natalensis* or SCID mice can be done in a range of immunosuppressed rodents.

1. Introduction

African sleeping sickness (or human African trypanosomiasis (HAT)) is endemic in 36 countries in sub-Saharan Africa. It is estimated that 60 million people are at risk of infection, that 500 000 people are already infected and approximately

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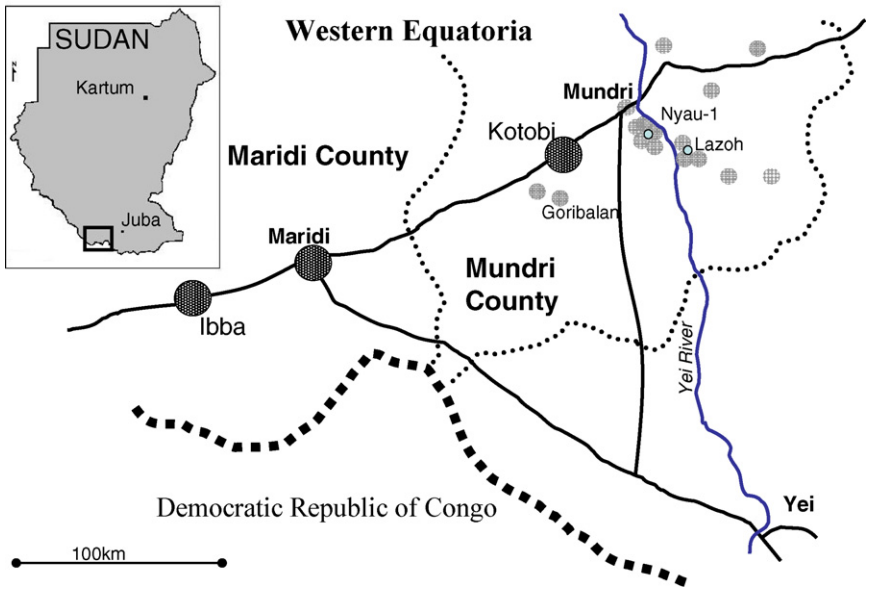

Figure 1 Study site in Western Equatoria in south Sudan. : the origin of the patients; : Médecins Sans Frontières laboratories.

50 000 die every year (WHO, 2004). The disease is caused by *Trypanosoma brucei rhodesiense* and *T. b. gambiense*. The former causes an acute disease in Eastern Africa, whilst *T. b. gambiense* generally causes a chronic disease in Western and Central Africa. In Sudan, HAT due to *T. b. gambiense* is highly endemic, with an estimated 1—2 million people at risk of infection and thousands of people infected.

Control of the disease relies mainly on chemotherapy. The drugs used in Sudan are pentamidine for first-stage disease and melarsoprol and eflornithine for second-stage disease. Melarsoprol is normally the first-line drug for treatment of second-stage disease. In recent years, high rates of melarsoprol treatment failure (>20%) have been reported in *T. b. gambiense*-endemic areas in Uganda, Angola and Sudan (Burri and Keiser, 2001; Legros et al., 1999; Moore and Richer, 2001). Additionally, a high fatality rate following melarsoprol treatment has been noted. This has led to a change of treatment policy and since September 2001 eflornithine became the first-line drug for second-stage disease in Ibba Hospital (Médicins Sans Frontières, 2002).

The role of resistant trypanosomes in treatment failures is not well understood, mainly because of a lack of *T. b. gambiense* isolates available from various endemic countries. One reason for this is the difficulty of isolating this trypanosome species and propagating it in laboratory rodents (Brun et al., 2001). The purpose of this study was to isolate *T. b. gambiense* from patients in Ibba Hospital and to evaluate a new cryopreservation medium, Triladyl® (Maina et al., 2006), under field conditions. Additionally, the capacity of different mouse breeds to develop a parasitaemia sufficient for laboratory investigations was studied.

2. Materials and methods

2.1. Isolation of trypanosomes from patients

Active screening of HAT patients was carried out by Médecins Sans Frontières France (MSF-F) in Mundri and Maridi counties, Western Equatoria, south Sudan (Figure 1). Diagnosis was done serologically (Magnus et al., 1978) or parasitologically (trypanosomes detected either in lymph node aspirate, venous blood or cerebrospinal fluid (CSF)). The number of white cells in CSF was determined and used for classification of the stage of disease (WHO, 1998). Patients who had been treated for HAT less than 12 months previously were considered relapses or treatment failures.

In the hospital, patients were informed of the objectives and protocol of the study. To be included in the study, the patients (or their guardians) had to give their consent (written/thumb print). The WHO and the Ministry of Health of Sudan People's Liberation Army (SPLA) approved the study protocol.

2.1.1. Cryopreservation of patient stabilates

Samples of 2—3 ml of venous blood were collected from patients into heparinised vacutainer tubes. Samples of 2 ml of CSF were also collected from two self-reporting patients attending the hospital. Trypanosomes in the samples were concentrated by centrifugation at 10 000 rpm for 10 min and the pellet was re-suspended in Triladyl® at a 1:1 ratio for blood and 1:10 for CSF. Aliquots of 500 μl were then transferred into Nunc® ampoules (Nunc, Roskilde, Denmark). Four to five stabilates were prepared per patient and stored in a liquid nitrogen dry shipper in the vapour phase at −150 °C. Dry shippers containing the stabilates were then transferred from the field sites to the Trypanosomiasis Research Centre (TRC) in Kenya where primary propagation in rodents was carried out.

2.2. Propagation of *Trypanosoma brucei gambiense* in laboratory rodents

2.2.1. Laboratory animals

Mastomys natalensis and Swiss White mice were bred at TRC. C57/BL and BALB/c mice, respectively, were purchased from the International Livestock Research Institute (ILRI)

and the Kenya Medical Research Institute (KEMRI), both in Nairobi, Kenya. Severe combined immunodeficient (SCID), C3H and FVB mice were purchased from the Charles River Laboratories, Inc. (Munich, Germany). NMRI mice were purchased from RCC Ltd. (Itingen, Switzerland). The animals were housed under conventional conditions and fed on commercial pellets and water ad libitum.

All animals (except SCID mice) were immunosuppressed by administration of cyclophosphamide at 300 mg/kg body weight applied intraperitoneally 1 day prior to infection and were treated every 10 days with 200 mg/kg.

2.2.2. Primary propagation in *Mastomys natalensis* and SCID mice

The presence and viability of trypanosomes in the stabilates was determined by inoculating one stabilate into one SCID mouse and two immunosuppressed *M. natalensis*. The stabilates were thawed rapidly in a water-bath at 37 °C and immediately injected intraperitoneally into the rodents. Parasitaemia was monitored every other day for 60 days by examination of tail blood using the haematocrit centrifugation technique (HCT) and/or the method of Herbert and Lumsden (1976). Parasitaemic animals were euthanised using CO_2 and blood was collected by cardiac puncture. The infected blood from the first passage was cryopreserved in Triladyl® and stored in liquid nitrogen. Successful stabilates were given an isolation code different from the original patient stabilate.

Rodents that remained aparasitaemic for 60 days after inoculation with cryopreserved material were euthanised and blood was collected by cardiac puncture. If no parasites could be detected using the HCT method, another two *M. natalensis* were inoculated with another duplicate stabilate.

2.2.3. Susceptibility of various strains of mice to *Trypanosoma brucei gambiense* isolates

The growth pattern of five randomly selected isolates was determined in C57/BL, BALB/c, Swiss White, NMRI, C3H and FVB mice as well as in *M. natalensis*. Prior to that, parasites from the primary propagation were passaged twice in *M. natalensis*. Trypanosomes were harvested from a donor *M. natalensis* and inoculated into groups of four animals (4×10^5 trypanosomes/mouse) of the different mouse breeds. Thawing of the stabilates, inoculation and parasitaemia determination were performed as described earlier.

2.3. Genotypic characterisation

Trypanosomes (passage 1) were purified using an anion exchange column (Lanham and Godfrey, 1970) and DNA was extracted using the Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN, USA) as previously described by Matovu et al. (2001a).

Amplification of the serum resistance-associated (*SRA*) gene and the *T. b. gambiense*-specific glycoprotein (*TgsGP*) gene was done as previously described by Radwanska et al. (2002a, 2002b). However, for both genes a nested PCR was performed. For *SRA*, the two sets of primers used were: SRA-out-s (5′-CCTGATAAAACAAGTATCGGCAGCAA-3′) and SRA-out-as (5′-CGGTGACCAATTCATCTGCTGCTGTT-3′); and SRA-inner-s (5′-ATA GTG ACA TGC GTA CTC AAC GC-3′) and SRA-inner-as (5′-AAT GTG TTC GAG TAC TTC GGT CAC GCT-3′). For the *TgsGP* gene, the primer pairs were: TbsGP-outer-s (5′-GCGTATGCGATACCGCAGTAA-3′) and TbsGP-outer-as (5′-GCTTCAACCGCCGCTGCTTCTA-3′); and TbsGP-s (5′-GCTGCTGTGTTCGGAGAGC-3′) and TbsGP-as (5′-GCCATCGTGCTTGCCGCTC-3′).

For control PCRs, the actin gene was amplified using primers act-s (5′-CCGAGTCACACAACGT-3′) and act-as (5′-CCACCTGCATAACATTG-3′). The amplifications were performed in 50 μl of reaction mixture containing PCR buffer (Qiagen, Basel, Switzerland), 200 mM of the four dNTPs, 1 μM of each primer and *Taq* polymerase in a PTC 200 Peltier thermocycler (MJ Research, Waltham, MA, USA) under the following conditions: initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 45 s, 62 °C (55 °C for actin) for 45 s and 72 °C for 120 s, followed by a final extension for 10 min and rapid cooling at 4 °C.

Eight microlitres of the PCR product were analysed by electrophoresis in a 1.25% agarose gel. The gels were stained with ethidium bromide (0.2 μg/ml) and visualised under UV light.

2.3.1. Sequencing of *TgsGP*

For sequencing, two independent PCR reactions were pooled, purified using QIAquick columns (Qiagen) and sequenced with the Big Dye Terminator kit (Applied Biosystems, Foster City, CA, USA). The products were run at the Computational and Molecular Population Genetic Laboratories (CMGP, Zoology Department, University of Bern) on an ABI sequencer.

3. Results

3.1. Patients

Blood samples were collected from 50 patients from Mundri (46), Maridi (2), Yambio (1) and Juba (1) counties (Figure 1). The mean age of the patients was 24 years (range 0.25—62 years) with >90% below 40 years of age. Most patients (49/50) were in the second stage of the disease, which was mainly based on a white cell count >5 cells/μl rather than the presence of trypanosomes in the CSF. Seven of the patients had previously been treated (7—12 months before): four with eflornithine (at 400 mg/kg/day for 14 days) and three with pentamidine (4 mg/kg/day for 7 days).

Forty-two patients were parasite positive in the blood and eight were aparasitaemic but card agglutination test for trypanosomiasis (CATT) positive at a 1:4 dilution. Samples of CSF were only collected from two patients attending the hospital for passive screening. For all other patients, a lumbar puncture had already been done in the field during active screening and for ethical reasons a second lumbar puncture was not performed in the hospital.

3.2. Primary propagation of stabilates from patients

Of the 50 blood samples inoculated into *M. natalensis* and SCID mice, 18 caused patent infections. The two CSF sam-

Table 1 *Trypanosoma brucei gambiense* isolates from Mundri county that could be propagated in laboratory rodents

Patient stabilate	Isolate no.	Patient data			Proof of infection and staging[a]			Primary propagation in rodents[b]			
		Bomas (village)	Age (years)	Sex	Blood/ lymph	CSF	CSF (cells/μl)	SCID mice		*M. natalensis*	
								Pre-patent period	Parasitaemia at freezing (days post infection)	Pre-patent period	Parasitaemia at freezing (days post infection)
I04008	Mundri 01	Midi	19	F	+	–	8	7	1.6×10^7 (7)	12	+++ (14)
I03037	Mundri 02	Amandi	12	F	+	–	49	7	1.6×10^7 (7)	8	+++ (10)
K03028	Mundri 03	Nyau 1	25	M	+	–	6	11	1.6×10^7 (12)	4	+++ (6)
K03048	Mundri 04	Buangyi	20	M	+	–	58	4	1.6×10^7 (6)	4	2.5×10^4 (5)
K03051	Mundri 05	Nyau 1	23	M	ND	–	50	7	8×10^6 (14)	23	++ (26)
K03030	Mundri 06	Goribalan	21	F	+	–	4	11	1×10^6 (21)	9	++ (11)
K03042	Mundri 07	Nyau 1	20	M	+	–	10	14	++ (14)	10	++ (12)
I04019	Mundri 08	Lazoh	10	M	+	–	17	13	+ (13)	21	3.2×10^7 (25)
K03043	Mundri 09	Wiri Lui	6	M	+	–	9	5	3.2×10^7 (9)	–	–
I04015	Mundri 10	Singo	12	M	+	–	104	11	1.6×10^7 (11)	–	–
K03045	Mundri 11	Nyau 1	12	M	ND	–	8	17	1.0×10^6 (22)	–	–
I04022	Mundri 12	Lazoh	9	F	+	–	8	16	2.5×10^4 (24)	–	–
K03010	Mundri 13	Mundri	27	M	+	–	61	–	–	3.5	8×10^6 (5)
I04021	Mundri 14	Gingo	23	F	+	–	45	–	–	18	2×10^6 (21)
I03030	Mundri 15	Lazoh	12	F	+	–	8	–	–	6	2.5×10^4 (7)
K03044	Mundri 16	Nyau 1	12	F	+	–	20	–	–	11	+++ (13)
K03032	Mundri 17	Singirigwa	22	F	+	+	783	–	–	17	+++ (18)
K03054	Mundri 18	Moto	25	M	+	–	22	–	–	11	+ (13)

CSF: cerebrospinal fluid; ND: not done.

[a] Trypanosomes were detected by examination of blood using haematocrit centrifugation technique (HCT) method and/or lymph node aspirate and by examination of CSF. Staging of disease was done by examination of CSF for the presence of trypanosomes and by white blood cell count in CSF (>5 cells/μl = second-stage disease).

[b] Primary propagation was done in immunosuppressed *Mastomys natalensis* and SCID mice. Parasitaemia in rodents was estimated by the method of Herbert and Lumsden (1976) or, for lower parasitaemia, by the HCT method.

ples did not lead to patent parasitaemia in the rodents. Patient stabilates that could be successfully propagated in the rodents were designated with the name 'Mundri' (the name of the county where all the patients came from) and a consecutive number; furthermore, they were termed isolates (Table 1). A slightly higher recovery was obtained in *M. natalensis* (14/18) compared with SCID mice (12/18). Only 8/18 stabilates could be propagated in both rodents. The stabilates had a similar pre-patent period in SCID mice (10.25 ± 4.26 days) and *M. natalensis* (11.25 ± 6.3 days). However, in *M. natalensis* only 3/14 stabilates developed a parasitaemia of $\geq 10^6$/ml, whereas in the SCID mice 9/12 stabilates reached that level of parasitaemia. Details of these 18 isolates are shown in Table 1.

3.2.1. Determination of the suitability of Swiss White mice for patient stabilates

Only five (I03030, I03037, K03048, K03045 and K03051) of the 18 patient stabilates that could be propagated in either immunosuppressed *M. natalensis* and/or in SCID mice caused patent infection in immunosuppressed Swiss White mice. The susceptibility of Swiss White mice was much lower compared with that of *M. natalensis*. The parasitaemias in Swiss White mice were generally low (apart from K03048) and could only be detected by the HCT method. For one stabilate (K03051), parasitaemia was sporadic and inconsistent.

3.2.2. Susceptibility of mice to secondary propagation of *Trypanosoma brucei gambiense*

All 18 isolates (Mundri 1 to 18, first passage) inoculated into immunosuppressed Swiss White mice caused patent infection, with a pre-patent period of 4 days. Most of the isolates (11/18) caused low and inconsistent parasitaemias ($<10^4$/ml), but the remaining isolates (Mundri 02, 03, 04, 06, 08, 14 and 17) grew to higher parasitaemia with levels $>10^6$ trypanosomes/ml (data not shown).

The growth patterns of five randomly selected isolates (passage 4) were compared in immunosuppressed C57/BL, BALB/c and Swiss White mice and *M. natalensis* (Table 2). The parasites were infective for all rodents used, with a similar pre-patent period of 3 days and a parasitaemia $>10^6$ trypanosomes/ml by Day 5. There was variation in the growth pattern between the isolates and between rodent breeds. For all five isolates, the highest parasitaemia ($>5 \times 10^7$ trypanosomes/ml) was noted in C57/BL mice. Overall, C57/BL and BALB/c mice sustained higher parasitaemias ($>10^6$/ml) for several days compared with Swiss White mice or *M. natalensis*. In all isolates, the parasitaemias dropped to undetectable levels by Day 16 post infection. Very few animals (4/80) died during the 60-day observation period. In NMRI, C3H and FVB mice, parasitaemia was always very low and far below the parasitaemia in C57/BL and BALB/c mice (data not shown).

3.3. Genotypic characterisation

Trypanosoma brucei subspecies-specific genotyping was performed by PCR using the *SRA* gene as a marker for *T. b. rhodesiense* (Radwanska et al., 2002a) and the *TgsGP* gene for *T. b. gambiense* (Berberof et al., 2001). The marker genes were amplified from genomic DNA by nested PCR

Table 2 Comparison of the growth characteristics of *Trypanosoma brucei gambiense* in different rodents. Animals were immunosuppressed with cyclophosphamide 300 mg/kg prior to infection. Each animal was inoculated with 4×10^4 trypanosomes (passage 3) by the intraperitoneal route. Parasitaemia was monitored every other day by examination of the tail blood for 20 days[a]

Isolate no. (patient stabilate)	Rodent breed	Period with parasitaemia $>10^6$/ml (mean duration) (days)	Highest parasitaemia (days post infection)
Mundri 02 (I03037)	C57/BL	4–16 (12.5)	2.5×10^8 (7)
	BALB/c	4–14 (4.25)	1.6×10^7 (8.5)
	Swiss White	5–7 (2)	0.8×10^7 (6)
	Mastomys natalensis	6–8 (2)	3.2×10^6 (7)
Mundri 05 (K03051)	C57/BL	5–9 (4.66)	5.0×10^7 (7.66)
	BALB/c	–	–
	Swiss White	5–16 (4.5)	1.3×10^7 (8.25)
	M. natalensis	6–7 (1.5)	4.6×10^6 (6)
Mundri 09 (K03043)	C57/BL	5–8 (3.75)	7.4×10^7 (6.75)
	BALB/c	5–9 (4.75)	6.4×10^7 (7.25)
	Swiss White	5–7 (3)	1.4×10^6 (6.5)
	M. natalensis	5–7 (3)	0.8×10^7 (6)
Mundri 13 (K03010)	C57/BL	5–14 (6.25)	1.5×10^8 (7.5)
	BALB/c	5–14 (6.26)	8.9×10^7 (7.5)
	Swiss White	5–7 (1.75)	3.2×10^6 (6.25)
	M. natalensis	5–7 (2.5)	1.1×10^7 (5)
Mundri 17 (K03032)	C57/BL	5–18 (13)	5.0×10^8 (14)
	BALB/c	6–9 (3.33)	5.0×10^7 (7.33)
	Swiss White	6–7 (2)	5.0×10^6 (6.5)
	M. natalensis	5–7 (3)	6.4×10^7 (7)

[a] Data shown are the mean of four animals.

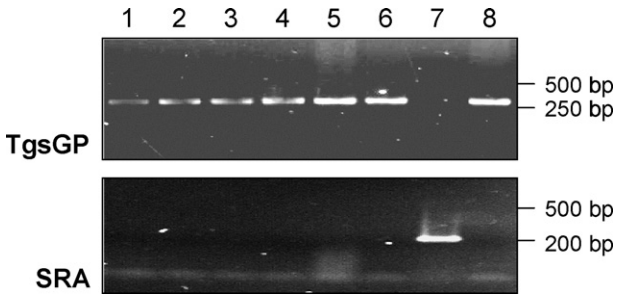

Figure 2 Genotypic characterisation: amplification of the *Trypanosoma brucei gambiense*-specific glycoprotein gene (*TgsGP*, top) and the serum resistance-associated gene (*SRA*, bottom) in trypanosomes isolated from human African trypanosomiasis patients. All 18 stabilates gave the same result, therefore only 6 of them are shown here (lanes 1—6). *Trypanosoma b. rhodesiense* STIB 704 (lane 7) and *T. b. gambiense* STIB 754 (lane 8) were used as reference.

and were analysed on agarose gels (Figure 2). All isolates tested were positive for *TgsGP* and negative for *SRA*. To verify that the correct gene was amplified, PCR products were directly sequenced. Apart from a few silent mutations, the obtained sequence (GenBank accession no. **DQ224158**) was identical to that published for *TgsGP* (GenBank accession no. **AJ277951**), confirming that the isolated trypanosomes were indeed *T. b. gambiense*.

4. Discussion

This is the first report of successful isolation of *T. b. gambiense* from patients in south Sudan. Eighteen viable isolates could be cryopreserved under field conditions using the new cryomedium Triladyl® (Maina et al., 2006). In previous studies, a low recovery rate or complete loss of *T. b. gambiense* populations following cryopreservation in medium containing 10% glycerol have been reported (Brun et al., 2001; Matovu et al., 2001b). The recovery rate achieved of 43% is very satisfactory and may be attributed to the superior cryopreservation medium Triladyl®.

Viability of the isolates in rodents depended on the degree of parasitaemia in the patients. Parasitaemia in patients was always low and only detectable by HCT; in eight patients parasites were not detectable at all although the patients were CATT positive. Earlier reports also noted the poor infectivity of *T. b. gambiense* for laboratory rodents, leading to a low isolation success (Aerts et al., 1992; Dukes et al., 1989).

In the current study, we used the African rodent *M. natalensis* (immunosuppressed) for primary propagation of the patient stabilates on a comparative basis with SCID mice, which have no functional T- and B-lymphocytes (Bosma et al., 1983). *Mastomys natalensis* was reported to be a better model for *T. b. gambiense* than rats and mice (Mehlitz, 1978) and has been extensively used for propagation studies (Aerts et al., 1992; Dukes et al., 1989; Gibson et al., 1978; Matovu et al., 2001b; Zillmann and Mehlitz, 1979). Inoue et al. (1998) first described the high susceptibility of SCID mice to *T. b. gambiense* isolates, a finding that was confirmed in our study. However, the use of SCID mice is restricted by the high cost of the mice as well as availability. On the other hand, breeding colonies of *M. natalensis* are already in place at institutes in East Africa.

Normal inbred or outbred laboratory mice have generally not been found to be susceptible to *T. b. gambiense* isolates, although they can easily be infected with *T. b. rhodesiense* trypanosomes. However, we found that following immunosuppression different breeds of laboratory mice could be infected with the second or third passage of our Mundri *T. b. gambiense* isolates. Even stabilates prepared directly from patients were infective for immunosuppressed Swiss White mice, although with a lower success rate (5/18) compared with *M. natalensis* (14/18) or SCID mice (12/18). However, after a second passage, all 18 isolates were infective for immunosuppressed Swiss White mice and 7 of them developed a parasitaemia $>10^6$/ml. In other mouse strains (C57/BL, BALB/c, NMRI, C3H and FVB), the parasitaemia was very low, except for the three isolates K03028, K03048 and K03043, which reached parasitaemias $>10^5$/ml in C57/BL and BALB/c by Day 14 post infection. Passaging the isolates at least three times increased their virulence, resulting in parasitaemias $>10^7$/ml.

In conclusion, we recommend that infected blood samples from *T. b. gambiense* patients should be cryopreserved in Triladyl® medium. Primary propagation and subsequent subpassage should be done in immunosuppressed *M. natalensis* or in SCID mice. Further subpassages can then be done in immunosuppressed laboratory mice (e.g. C57/BL or BALB/c). Available mouse breeds should be screened following immunosuppression with cyclophosphamide at 300 mg/kg prior to infection and repeated once per week at 200 mg/kg. This propagation scheme is efficient as well as economical. However, it must be kept in mind that repeated passage through rodents can lead to parasite strain selection, thus the results obtained must not be interpreted as representing the true strain diversity in the human population. The proposed scheme will allow the characterisation of *T. b. gambiense* from various hot spots of Central Africa and therefore facilitate the monitoring of drug-resistant trypanosomes. These new *T. b. gambiense* isolates from Sudan are a unique source for further scientific investigations. Their characterisation, especially for drug sensitivity, will help to elucidate the cause of the high melarsoprol treatment failure rates reported from the area.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

Authors' contributions

NWNM, JMN and RB designed the study outline; NWNM and MO carried out the fieldwork; NWNM, CO and CK carried out the animal experiments; PM supervised the genotypic characterisation; NWNM and RB drafted the manuscript. All authors read and approved the final manuscript. NWNM and RB are guarantors of the paper.

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