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Part I: PROTOCOLS FOR ISOLATION, DRUG SENSITIVITY AND CHARACTERIZATION

ISOLATION OF *T.B GAMBIENSE* FROM PATIENTS

Rationale

T.b gambiense form of Human African Trypanosomiasis is characterised by chronic disease and low parasitaemia. In order to study and further characterize this parasite it is important to isolate and preserve these parasites to maintain their genotypic and phenotypic characteristics. This could help in epidemiological studies especially in mapping out the drug resistant strains and therefore improve in the management of the disease. EANETT member countries of Kenya, Uganda, Sudan, Tanzania and recently invited Democratic Republic of Congo have come up with the following isolation protocol for *T.b gambiense*

Important key parameters and prerequisites

In order to carry out this protocol one must have the following:

- Gradual freeze of the trypanosomes using liquid nitrogen
- Providing appropriate cryo-medium to maintain the integrity of the cells
- Maintaining an appropriate rodent/mice colony for propagation of the parasites

Cryopreservation of blood samples

- Use CATT screening test to identify suspected patients
- Take lymph aspirates for patients with palpable lymph nodes and capillary blood from the rest of the CATT positive patients
- For patients who are parasite positive, take 4-5 mls of blood using heparinised vacuitainer tubes
- Distribute the blood into 3 microtubes
- Label the tubes accordingly
- Centrifuge 12,000 rpm for 5 minutes
- Take the buffy coat using a Pasteur pipette
- Put equal portions of 0.25 ml of buffy coat in cryotubes and add equal volume of cryo-medium Triladyl to make a total of 0.5ml.
- Mix the buffy coat and the cry-preservative gently using Pasteur pipette
- Store the samples in liquid nitrogen suspending the samples in liquid Nitrogen vapour for one hour followed by dipping of tubes containing the parasites in the liquid nitrogen tank.

Preparation of Triladyl medium:

- Collect egg yolk with syringe through punctured egg shell, cleaned withalcohol, into a graduated beaker
- Mix well
  - 3 vol. of phosphate Buffered Saline Glucose
  - 3 vol. of Triladyl
  - 1 vol of egg yolk
- Divide in small aliquots
- Keep frozen at -20C
- Thaw aliquot before use
- Note: work as sterile as possible

Cryopreservation of CSF samples.

- Take 5ml of CSF from the lumbar region
- Put in plain vacutainer tube.

- For diagnosis take a drop of the CSF and put in Haemocytometer chamber and look for the parasites. If there are no parasites seen, concentrate the parasites by putting 2mls in a sealed Pasteur pipette and centrifuging at 3,000 rpm for 5 minutes. Examine for the parasites using 10x at the sediments.
- For the positive samples, proceed as follows:
- Use Pasteur pipette to distribute the CSF from one patient in 3 cryo-tubes
- Centrifuge in a microcentrifuge at 12,000 rpm for 5 minutes
- Use Pasteur pipette to withdraw the supernatant from each tube
- Add equal volume of the prepared Triladyl to the sediment
- Mix with Pasteur pipette
- Preserve from each patient have three tubes
- Put the samples in socks and put in liquid nitrogen vapour for one hour before dipping in liquid nitrogen

Propagation in animals

- Use of immunosuppressed *Mastomys natalensis*-a single dose of 200 mg/Kg bd wt, 250mg/Kg bd wt or a total dosage of 300mg/Kg bd wt given as a split dose a day prior infection
- Grammomys surdaster
- SCID mice
- Other rodent spp available

Required investigations and action.

- Member countries to acquire enough stocks of Triladyl
- Member countries to adopt preservation of buffy coat instead of the whole blood

Adaptation and propagation in culture of bloodstream forms of *T. b. gambiense*

- Cryopreserved *T. b. gambiense* stabilates are propagated in immunosuppressed *Mastomys*
- Trypanosomes are harvested by cardiac puncture from infected animals using heparin as the anti-coagulant.
- The bloodstream forms are initiated into culture by obtaining the buffy coat
- The trypanosomes are then transferred into a T25cm^3 flask containing *Mastomys* embryo fibroblast feeder layer cell line
- Cultures are **monitored daily** for presence of parasites and media replaced appropriately at least every second day
- The trypanosomes started on feeder layer can then be adapted to axenic conditions within four weeks by transferring trypanosome containing supernatant to flasks without feeder layer cells.
- The flasks are incubated at 37ºC in 5% CO_2 atmosphere.

Culture medium

MEM, supplemented with 25mM HEPES, 1g/l additional glucose, 2.2g/l $NaHCO_3$ and 10ml/l MEM non-essential amino acid (100x).

The medium is further supplemented according to Baltz

0.2mM 2-mercaptoethanol, 2mM Na-pyruvate, 0.1mM hypoxanthine and 0.016mM Thymidine, 15% normal human serum and 5% heat inactivated foetal calf serum

DRUG SENSITIVITY DETERMINATON

Drug sensitivity determination with non-culture adapted isolates

Rationale

It is important to know the drug sensitivities of field isolates (primarily melarsoprol and new clinical candidates)
Do the assay with a population as close to the patient as possible to avoid selection
Culture adaptation is a time and resource consuming process

Important parameters

1. Trypanosome number
2. Culture medium and serum; medium without additional hypoxanthine
3. Culture conditions (pH, temp 37°C, 5% CO_2)
4. Assay duration 40 hours
5. Media without hypoxanthine

Protocol: In vitro ^{3}H-Hypoxanthine incorporation assay with *Trypanosoma* spp. bloodstream forms

- Put 50 µl of medium (Baltz medium or Hirumi without hypoxanthine and thymidine but with a serum component) into all wells of rows A to G of a 96 well plate, but add 100 µl to the background wells.

CONTROLS BACKGROUND

A												
B												
C												
D												
E												
F												
G												
H												

DRUG 1 Drug 2 DRUG 3 DRUG 4 DRUG 5 DRUG 6

- Put 100 µl of medium containing two times the highest drug concentration to be tested into wells of row H (column 1: drug No 1, column 3: drug 2...).
- Prepare serial dilutions using a multipipette by transferring 50 µl from the wells of row H into wells of row G and mix by pipetting the medium 5 times.
- Continue with the dilution from bottom to the top and discard the remaining 50 µl from wells of row B. This will lead to a 2-fold serial drug dilution.

- Add to each well 50 ul of trypanosomes suspension (2x 10^6/ml) except for the background wells. Final concentration 10^5/ well
- Incubate plate for 24 hours at 37℃, 5% CO_2.
- Prepare the ^{3}H-Hypoxanthine stock solution in medium to get a final concentration of 1uCi/well (1µCi /20 µl medium) and add the label to each well
- Incubate another 16 hours at 37℃, 5% CO_2
- Harvest the trypanosomes with a cell harvester on a glass fiber filter (Whatman grade 934 AH; Cat No. 1827849) and air dry the paper completely.
- Count the filter papers in a liquid scintillation counter. Subtract the mean background from each individual count and express the cpms as percentage of the control.

Assay medium

MEM (with Earle's salts) + additional 1 g/l D-glucose
10 % heat inactivated normal human serum + 5% FBS
1 % 2-Mercaptoethanol
1 % Hirumi stock solution

Preparation of Hirumi stock solution (100 x final conc.):

100 mM Na-Pyruvate: (MW 110.05)	11.0 mg/ml
5 mM Bathocuproindisulfate (MW 564.5)	2.82 mg/ml
150 mM L-Cysteine·HCl (MW 157.6)	23.6 mg/ml
200 mM L-Glutamine (MW 146.1)	29.3 mg/ml

Dissolve all ingredients in distilled water. If solution precipitates, add NaOH until it is clear again. Adjust pH to 9.7. Filter sterilize through 0.2 µm filter and store in small aliquots at -20℃.
Recommended highest drug concentrations to be used:
Melarsoprol: 50 ng/ml
Pentamidine: 50 ng/ml
DB75: 100 ng/ml
Diminazene: 50 ng/ml
Nifurtimox: 10 µg/ml

Required actions

Test the various Baltz medium components (thymidine, bathocuproinesulfonate, L-cysteine) and serum supplements to optimize for best growth. Will be done at STI.

Drug sensitivity determination with culture adapted isolates

Culture adapted isolates allow longer durations of assays and selection of possible end points. For instance Metabolic - pyruvate assays and fluorochrome- Alamar blue and pico green. In these assays IC_{50} can be calculated.
Pico green - new upcoming assay method under test in STI. Very sensitive and can use same fluorometer as Alamar blue with an appropriate set of filter cubes.

Other end points such as minimal inhibitory concentration, MIC, could also be used. However MIC determination is subjective (individual) and thus results are not always reproducible. On the other hand counting methods have limitations, they are time consuming and wells not assessed at the same time.
Need - Fluorometer with appropriate filter set

1. In vitro Alamar blue assay with Trypanosoma spp. bloodstream forms

Rationale

It is important to know the drug sensitivities of field isolates (melarsoprol and new clinical candidates)
The Alamar blue assay can be used if culture adapted.
Alamar blue is cheaper and non- radioactive as compared to H^3-hypoxanthine.
The pyruvate method is not suited as a plate reading assay since it is very labour intensive and not recommended for a plate system format.

Important parameters

Trypanosomes seeding number not too high or too low
Time 70 hours and incubate for further 2-4hours.

Protocol: In vitro alamar blue assay with *Trypanosoma* spp. bloodstream forms

- Put 50 µl of medium (Baltz medium or Hirumi) into all wells of rows A to G of a 96 well plate, but add 100 µl to the background wells.

A											
B											
C											
D											
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H											

DRUG 1 Drug 2 DRUG 3 DRUG 4

- Put 100 µl of medium containing two times the highest drug concentration to be tested into wells of row H (column 1: drug No 1, column 3: drug 2...).
- Prepare serial dilutions using a multipipette by transferring 50 µl from the wells of row H into wells of row G and mix by pipetting the medium 5 times.
- Continue with the dilution from bottom to the top and discard the remaining 50 µl from wells of row B. This will lead to a 2-fold serial drug dilution.
- Add to each well 50ul of trypanosomes suspension ($4x\ 10^4$/well) except for the background wells.
- Incubate plate for 70 hours at 37°C, 5% CO_2.
- Add 10ul of Alamar blue (resazurin 12.5mg in 100ml PBS)
- Incubate another 2-4 hours at 37°C, 5% CO_2
- Read the plates at a fluorometer at 530nm excitation and 590nm emission wavelength
- IC50 determination [in more detail]

Recommended highest drug concentrations to be used:
Melarsoprol: 50 ng/ml

Pentamidine: 50 ng/ml
DB75: 100 ng/ml
Diminazene: 50 ng/ml
Nifurtimox: 10 µg/ml

2. In vitro Pico green with *Trypanosoma* bloodstream forms

To be established by the STI group.

3. Long term feeder layer assay (6-10 days)

Rationale

Longer assay duration means that trypanosomes are more affected by the drugs and thus a clear endpoint can be expected

Important parameters

Change media and drug every day
Good feeder layer system required (*Microtus montanus* or *Mastomys natalensis* feeder cells)

Protocol

- Put 250ul of cell suspension of feeder layer cells of *Mastomys* embryo fibroblasts at a density that will reach confluency in 24 hours (this should be determined in an earlier study)
- Remove 200ul of media after 24 hour incubation.
- Bloodstream forms are seeded onto the feeder layer in 48 well plates in serial drug dilutions ranging from 72 to 1.125 ng/ml.
- Cultures are incubated at 37°C in a humid atmosphere containing 5% CO_2.
- Every other day the cultures are monitored and medium replaced with fresh medium containing the appropriate drug concentration.
- After 6 days the minimum inhibitory concentration (MIC) is determined microscopically.

A							
B							
C							
D							
E							
F							

Controls

DRUG 1 Drug 2 DRUG 3 DRUG 4

In vivo drug assay protocol for *T. b. gambiense* and *T. b. rhodesiense*

Important parameters for *T.b. gambiense*

Rodent: test rodents available and choose most susceptible
Immunosuppression prior to infection and every 10 days with cyclophosphamide at 200mg/kg bwt for a maximum of 3 times
Check parasitaemia using the haematocrit centrifugation method.

Protocol

- Ensure rodents have adequate acclimation period (4-8days)
- Infect rodents (inbred or out bred white mice) with 1 X 10^5 tryps/ml i.p. (should check the virulence of isolate in a pilot study first) 4-5mouse/group including a control group for each isolates
-
- Start treatment 3-6 days post infection. Treatment should be i.p. for most drugs unless otherwise indicated. For melarsoprol dosages should range from 4 x 0.5 to 4 x 20mg/kg bwt.
-
- Check parasitaemia by wet smear twice a week the first two weeks then once a week for 60 days
-
- Data interpletation
  - cure negative for 60 days
  - not cured – positive anytime during the experimental period. Express as cured/ infected

Reference drugs

A small panel of reference drugs should be used as an internal standard. The following drugs in the following form are recommended:

Melarsoprol, Arsobal from Aventis as 5 ml ampoules with 3.6% melarsoprol in propylene glycol, from WHO

Diminazene diaceturate from SIGMA (Cat. No.)

Pentamidine isethionate from SIGMA (Cat No.)

DB75 synthesised by Dr. D. Boykin, Atlanta; can be obtained from STI or from TRC

Nifurtimox can be obtained from STI

Preparation of stock solutions

Drugs which come in powder form are being dissolved in 100% DMSO at 10 mg/ml. Aliquots are frozen at -20°C or lower. On the day of the assay or mouse experiment working solutions are being prepared in culture medium. This working solution can be frozen once but not several times. Melarsoprol is directly diluted in water and for the final dilution step in culture medium and this has to be prepared every day.

Reference isolates

Reference isolates are needed to compare the sensitivity of new isolates to existing ones with known characteristics. Ideally we should have a panel of sensitive and of resistant isolates as far as resistant ones are available. They also can act as internal control i.e. similar IC_{50} values should be obtained by experienced laboratories. It is not meant to have all of these isolates as references in each assay. Other isolates, especially recent isolates and preferably drug resistant ones, can be used as long as they show stable and documented characteristics.

T.b.brucei STIB795 (=S427 strain); for vitro and for vivo
T.b.rhodesiense STIB900 (=STIB704) from Tanzania; for vitro and for vivo
T.b.rhodesiense EATRO243; for vivo (not adapted to axenic culture)
T.b.gambiense STIB930 (=TH1- …, Cote d'Ivoire); for vitro and vivo
T.b.gambiense K03048, South Sudan; for vitro and vivo
T.b.gambiense DAL898R, Cote d'Ivoire; for vitro and vivo

Data interpretation

Data produced by different laboratories differ from each other even when using the same protocol. There is always variation in the parameters and the biological materials used, and in addition a bioassay has an intrinsic variation. Therefore, it is not reasonable to define general threshold to be used by different laboratories. Each lab has to establish its own data set using isolates known to be sensitive and others which origin from refractory patients. But even such isolates may not show a higher IC_{50} value when tested in vitro. If this is the case and isolates with a reduced in vitro sensitivity are missing, one can at least define a threshold for sensitivity. In vitro this is an IC_{50} value and isolates with an IC_{50} below this value are considered sensitive. In the mouse model the threshold is a treatment regime (e.g. 4 day treatment at 2 mg/kg i.p.) which cures sensitive isolates. Once an isolates cannot be cured by this minimal curative dose, then higher doses (2x and 4x the original dose) have to be applied and the outcome measured.

Once more data on in vitro and in vivo experiments by different laboratories using the same (or a similar) protocol are available, better criteria for data interpretation can be worked out.

TRYPANOSOME CHARACTERISATION

Rationale

Differential diagnosis of infecting trypanosomes for purposes of identifying the subspecies (*T. b. gambiense* vs *T. b. rhodesiense*) and identification of animal reserviors of sleeping sickness. Detection of potentially drug resistant isolates by P2 adenosine transporter characterisation.

DNA isolation

Purpose: to describe procudure for isolation of reliable quality DNA from patient or infected animal blood for subsequent characterisation using the PCR.
2 methods suggested namely

- FTA cards from
- Puregene DNA isolation Kit (Gentra systems)

1. FTA-cards

Rationale

The FTA card (Whatman) has been designed with special properties for long-term preservation of DNA in blood spotted on the matrix. This gives advantage over ordinary filter paper in that the DNA remains intact prior to extraction.

Critical parameters and prerequisites

- Volume of blood (at least 200µl per spot area)
- A better yields is obtianed if the buffy coat instead of whole blood is spotted on the card
- Storage conditions (silica gel to prevent moisture from damaging DNA quality)

Procedure

- Take a 3mm sample from a dried blood spot on the a FTA card (Whatman) and put in a
- centrifuge tube
- 2. Add 200µl of FTA purification reagent.
- Incubate for 5 minutes at room temperature while mixing.
- Remove all FTA reagent .
- Repeat 3 and 4 twice.
- Add 200µl of TE buffer (10mM MTris-HCl, 0.1mM EDTA pH8.0).
- Incubate at room temperature for 5 minutes.
- Remove all the TE.
- Repeat 6-8 for 2 washes.
- Dry the discs at room temperature for 1 hour or at 56^{0}C for 10 minutes before PCR

2. Puregene DNA isolation Kit

Rationale

The Puregene DNA isolation kit has proved to be efficient for recovery of DNA from low parasitaemic samples characteristic of *T. b. gambiense* infections.

Critical parameters and prerequisites

- Volume of blood (at least 500µl per extraction)

- Blood collected in EDTA may yield better quality DNA
- Avoid continued freeze-thawing of unprocessed blood

Procedure

- To 1500□l of RBC lysis solution in an eppendorf tube, Add 500□l of blood (preferably collected in EDTA tubes) and mix gently by inverting several times.
- Incubate at room temperature for 10 minutes, followed by centrifigation at 13000g for 5 minutes. Discard the supernatant.
- Add 300□l of cell lysis solution containing 100□g/ml proteinase K and resuspend the pellet by pipetting
- Incubate at 55 ^{o}C for 1 hour
- Add 200□l of protein precipitation solution and mix gently by inverting. Incubate on ice for 5 minutes.
- Centrifuge at 13000g for 5 minutes and collect the supernatant into a sterile Eppendorf tube.
- Add 400 µl of isopropanol to the collected supernatant. Mix gently as you observe for precipitating DNA.
- Spin at 13000g for 5 minutes and discard the supernatant. Wash the DNA pellet (you may not see it!!) with 70% ethanol.
- Spin once again, discard supernatant and allow to air dry for 15 minutes.
- Dissolve the dry pellet in 20 µl DNA hydration solution and allow to rehydrate at 65 ^{0}C for 1 hour, or overnight at room temperature.
- Use up to 5µl of the rehydrated DNA for the PCR

Note: The same protocol may be used to prepare DNA from Cerebrospinal fluids. In this case the process starts at step 3, with addition of 50µl of CSF or CSF pellet to 250µl of the cell lysis solution.

Characterisation of *T. brucei* sub species

Primer sets

TBR primers for the brucei group (Moser et al., 1989)

TBR primers for the brucei group (Masiga et al., 1992)

TBR-1 5'GAATATTAAACAATGCGCAG3'
TBR-2 5'CCATTTATTAGCTTTGTTGC3'

SRA primer for *T. b. rhodesiense* (Gibson et al., 2002)
SRA-A 5'GACAACAAGTACCTTGGCGC3'
SRA-E 5'TACTGTTGTTGTACCGCCGC3'

TgsGP for *T. b. gambiense* (Radwanska et al., 2002)
TgsGP-F 5'GCTGCTGTGTTCGGAGAGC3'
TgsGP-R 5'GCCATCGTGCTTGCCGCTC3'

Table-1: The buffer composition and quantity for one Sample

Materials (1rxn)	TBR_{1-2}	SRA_{A-E}	$TgsGP_{FOR-REV}$
10x buffer	1x	1x	1x
$MgCl_2$	1.5mM	1.5mM	1.5mM
dNTPs (10mM)	200µM	200µM	200µM
Specific primer-1(100Pmol)	2.5pM	2.5pM	2.5pM

Specific primer-2(100Pmol)	2.5pM	2.5pM	2.5pM
Taq DNA polymerase (2.5 unit/)	1.25U	1.25U	1.25U
Template DNA	-	-	-
Deionized water	-	-	-
Total	20µl	20µl	20µl

PCR conditions

PCR reaction conditions for TBR-primers:
Initial denaturation 94°C for 3 minutes,
30 cycles of:
Denaturation 92-94°C for 30 seconds,
Annealing 60°C for 45 seconds,
Extension 72°C for 45 seconds,

And final extension 72°C for5 minutes.
Expected product size 177bp (Moser et al., 1989)

PCR reaction conditions for SRA-primers:
Initial denaturation 95°C for 3 minutes;
30-35 cycles of:
Denaturation 95°C for 30 seconds,
Annealing 60°C for 30 seconds,
Extension 72°C for 60 seconds;

And final extension 72°C for 5 minutes.
Expected product size 460bp (Gibson et al., 2002)

PCR reaction conditions for TgsGP-primers:
Initial denaturation 95°C for15 minutes;
45 cycles of:
Denaturation 94°C for 60 seconds,
Annealing 63°C for 60seconds,
Extension 72°C for 60 seconds;

And final extension 72°C for 5 minutes.
Expected product size 308bp (Radwanska et al., 2002)

Trypanosomes of animal origin

ITS primers for differential diagnosis of *T. brucei, T. congolense. T. evansi* and *T. vivax*
Being evaluated by the IAEA for formulation of an ITS diagnostic kit
ITSC-F 5’CCGGAAGTTCACCGATATTG3’
ITSB-R 5’TTGCTGCGTTCTTCAACGAA3’

Materials (1rxn)	**Final concn.**
10x buffer	1x
dNTPs (10mM)	200µM
$MgCl_2$	1.5mM
ITSC-F	0.2µM
ITSB-R	0.2µM

Taq DNA polymerase (2.5 unit)	1.5U
Template DNA	
Deionized water	-
Total	25µl

PCR conditions

Initial denaturation 95^{o}C for5 minutes;
40 cycles of:
Denaturation 95^{o}C for 1 minute,
Annealing 60^{o}C for 1 minute,
Extension 72^{o}C for 1 minute;

And final extension 72^{o}C for 10 minutes.

Expected product sizes
T. brucei/evansi 580bp; *T. vivax* 280bp; *T. congolense* (Kilifi) 650; *T. congolense* (Savanna) 700bp; *T. congolense* (Forest) 710bp (Desquesnes and Davilla, 2002)

P2 adenosine transporter (TbAT1)

Purpose: The P2 adenosine transporter is thought to contribute to treatment failure following melarsoprol for late stage gambiense sleeping sickness. Determination of P2 genotype will be useful to generate more evidence for its involvement in treatment outcome.

Two primer sets are used for amplification of the gene
ATF-1 (5'-GAAAGCTTAATCAGAAGGATGCTCGGGTTTGACTCA-3')
ATR-1 (GAGGATCCTGAACAGTATTCGTATGACGATTAGTGCTAC-3') which anneal at the 5' and 3' non-translated regions of the TbAT1 gene (Matovu *et al*. 2001).
The final PCR reaction mix contains 1.5mM Magnesium chloride, 0.2mM dNTPs, 25 pmoles of each of the primers, 1× PCR buffer and 0.03 units/ µl of RED Taq DNA polymerase (Sigma) in a total reaction volume of 25µl.

<u>The PCR amplification conditions:</u>
Initial denaturation at 94^{0}C for 5 minutes,
30 cycles of:
Denaturation at 94^{0}C for 1 minute,
Annealing at 55^{0}C for 1 min
Extension at 72^{0}C for 2 minutes.

And final extension at 72^{0}C for 7 minutes
Expected product 1400bp. This may not be observed on the gel due to scanty parasitaemia in patient blood, a second PCR using the first product as template may be done with primers:
Sfa-s (5'-CGCCGCACTCATCGCCCCGTTT-3')
Sfa-as (5'-CCACCGCGGTGAGACGTGTA-3').

<u>Amplification conditions</u>
Initial denaturation 94^{0}C for 5 minutes

30 cycles of:
Denaturation at 94^{0}C for 1 minute,
Annealing at 65^{0}C for 1 minute
Extension at 72^{0}C for 1 minute.

And final extension at 72^{0}C for 7 minutes
Expected product 677bp

SfaNI RFLP analysis

The 677 bp fragment of the TbAT1 gene amplified above is digested with SfaN1 (New England Biolabs) at 37^{0}C in the presence of NEBuffer 3.0 (New England Biolabs) for 5 hours. This is followed by gel electrophoresis on 2.5% agarose gels.

Expected fragment sizes
566bp and 111 bp for wild type; 435bp and 242bp for mutant P2 transporter.
Note: SfaNI has a low restriction efficiency such that even after digestion, the 677bp band still appears on the gel.

Allele-specific PCR for detection of Mutant P2 adenosine transporter gene.

Rationale

The current method of mutant P2 adenoseine transporter (TbAT1) detection invoves use of the PCR to amplify the region of interest, followed by restriction digestion with SfaNI enzyme to detect mutant TbAT1. SfaNI is, however one of the most expensive restriction enzymes on the market. The allele specific PCR circumvents this problem by use of a multiplex PCR in which one of the primers is specific for mutant TbAT1.

3 Primers for use in a single PCR:
Sfa-s 5'-CGCCGCACTCATCGCCCCGTTT-3'
Sfa-as 5'-CCACCGCGGTGAGACGTGTA-3'
Sfa-mut 5'GGACATGGACCAGGTGGAAGG3'

PCR conditions:
Initial denaturation 94^{0}C for 5 minutes
30 cycles of:
Denaturation at 94^{0}C for 1 minute,
Annealing at 64^{0}C for 1 minute
Extension at 72^{0}C for 1 minute.

And final extension at 72^{0}C for 7 minutes
Expected product 677bp in all cases; and an additional 271bp if mutant TbAT1 is present

Required investigations, actions

Required activity	Actions
Field evaluation of the molecular techniques Risk of overlap of rhodesinese and gambiense HAT Animal reservoir of *T. b. gambiense* Drug sensitivity	Assess their diagnostic sensitivity vs analytical sensitivity Differential diagnosis in Uganda, Tanzania and Sudan Evaluation of Allele-specific PCR for detection of mutant TbAT1 and correlation with phenotype

Part II: PROTOCOLS FOR VECTOR, ANIMAL AND HUMAN SAMPLING IN HUMAN AFRICAN TRYPANOSOMIASIS

GENERAL ISSUES CONSIDERED IN SAMPLING

Before commencement of any study that requires sampling, a basic decision must be reached about the population of interest. For example in case of tsetse sampling, one may be interested in tsetse (of all species) found in a given belt. An understanding of the population of interest will enable selection of appropriate tools for sampling. The same applies to epidemiological studies. A proper definition of the research question will determine the tools to be used and how the work is to be done in order to generate the required data.

Because it is usually not possible to screen the whole (vector, human or animal) population, only part of it or *sample* will be obtained. Data or any information generated from this sample will be used to make statements or inferences about all the flies, animals or humans in the population, including population density, infection rates etc.

The main objective in sampling is therefore to select, at reasonable cost, a sample size that is as representative as possible of the entire population. A representative sample must be obtained at random - that is, where each unit of the population has equal chance of selection. Techniques used in obtaining this sample must be such that the size of the sample should be of a certain minimum size in order to confidently make statements about the population as a whole. There are several sampling principles that should be taken into consideration in order to obtain a representative sample. These include:

Stratification

Stratification of the population is the process of dividing the population into relatively homogenous subgroups called *strata*, and then taking samples from each strata. In the case of tsetse sampling, stratification may take the form of subdividing the belt into zones of homogenous vegetation, and then sampling from each zone. For example, suppose inference is to be made on tsetse population and distribution in a tsetse-belt found in a given ecosystem using data generated from a selected sample; further investigations need to be made to determine whether this ecosystem can be delineated into sub-ecosystems (e.g., containing of different vegetation types, different tsetse host types, etc). In view of this, it is important to stratify this ecosystem into the suitable sub-ecosystems, and give a good basis for this stratification before embarking on sampling.

It is convenient to stratify as far as possible using the given domains of interest for which the sampling is required to address. These can be stratification either by tsetse species, by vegetation type or host type, etc.

Random Sampling Procedures

Random sampling is a selection procedure, which ensures that every unit of the population or strata of the population has equal chance of being selected. Random sampling is best done with a table of random numbers such as those usually found in a statistics text book or through drawing of numbers written on a paper or cardboard squares 1cm x 1cm, from a container. A computer can also generate these numbers. In tsetse sampling, for example, it might be difficult to position trapping devises throughout the delineated strata. These homogenous strata can thus be subdivided into blocks (based on a given criteria such as administrative locations, villages, etc.). A list of all these manageable blocks should then be drawn from which a suitable number from which a set of sampling devises can be made. For example, it may not only be important to make a list of all villages, or all locations but also obtain data on the approximate size (areas) of each block.

Sample Size

This will involve determining the number of blocks/villages per stratum and how many sampling devices (e.g. traps) per block should be set. In case of blocks, a representative sample must not only be random, but must also be large enough to reflect all blocks in a stratum. The important point to consider is that ***'sample size will depend on the variability within each given population and not on size of population'***. Therefore the number of blocks/villages within a stratum will depend on variability within the strata, and the number of sampling devises set in a block will also depend on variability within this block. Domains or issues of interest to the researcher will describe variability.

Another important aspect is that sample size must conform to the time and cost constraints of the study/survey. The major cost of such a survey include the fixed costs of developing the sampling tools (traps), training of assistants, and of developing a suitable sampling method. The marginal costs of including additional blocks in the sample may vary and in some cases may be relatively low. Therefore, this calls for ingenuity on the part of the researcher to determine when to increase sample size, particularly when there is doubt about the adequacy of the sample size for representing some variables.

HUMAN SAMPLING

The purpose of human and animal sampling is to determine the distribution and determinants of health related states or events in a specified population. The information generated is then used to prevent and control the health problems. Following selection of a sample as previously described, clinical examination is undertaken. For purposes of this protocol, we shall focus on sampling for HAT.

Clinical examination

Information on HAT suspects should be collected in data sheets during the examination. The data sheet may include but showed not be limited to the information given below.

- Study title
- Date
- Name of person (HAT suspect)
- Ethnicity
- Origin of person (HAT suspect)
- Village-village heads, relevant local authority
- Location/Parish/Camp
- Age of person
- Sex
- Clinical History
- Diagnosis
- Any diagnosis attended
- Pricking-how many times
- Lumbar Puncture done-how many times
- Treatment history

Diagnostic results

Date	CATT	¼ dil	1/8 dil.	Lymph node aspirates	HCT	mAET	Follow-up

- Name and Signature of the Examiner

1. Screening test for gambiense sleeping sickness following clinical examination

- Blood is collected in heparinized capillary tube from finger prick
- CATT test is performed **according to WHO guidelines**.
- In low endemic areas it may be necessary to dilute blood to enhance sensitivity.
- 50µl of CATT buffer is placed in 3-4 wells and 50µl of blood added to the first well and a double dilution done by transferring 50µl to subsequent wells.
- Precipitation at ¼ dilution or above is considered CATT positive. CATT positive cases with swollen lymph node are subjected to microscopic examination of a wet mount of lymph node aspirate for trypanosome detection.
- CATT positive cases without swollen lymph node are subjected to HCT for trypanosome detection.
- All HCT negative cases are screened using miniature Anion-exchange chromatographic technique (mAECT).
- All parasitologically positive individuals from the above tests are subjected to lumbar puncture for stage determination and appropriate treatment.

- All CATT positive but parasitologically negative cases are discharged and advised to return for follow-up at intervals of 2-3 months for a period of 1-2 years.

Serological technique

CATT/PL/FP-LATEX/PL

- 2-3 ml of whole blood from CATT positive at ¼ or above dilution, is collected in heparinized tubes, and approximately 0.5 ml is spotted on filter paper evenly to be eluted for CATT. The remaining blood is centrifuged and plasma collected for CATT and Latex.
- All positive for CATT and Latex are subjected to the standard parasitological tests for trypanosome detection. The negative ones are discharged.
- It is advisable to carry out CATT/PL/FP-LATEX/PL on 10% randomly selected negative individuals for CATT ¼ dilution.
- Note:
- CATT/DB is only carried out in areas of low endemicity.

2. Screening for Rhodesiense sleeping sickness

- Prick the finger of the HAT suspect and fill 3 capillary tubes with blood
- Prepare thick and thin smear for examination
- All individuals screened using HCT
- Positive HCT undergo lumbar puncture
- Two ml aliquot of venous blood is divided into three portions of 0.5ml each. One portion is inoculated into two mice. The second portion is cryopreserved (SOPs). The last portion is lysed in buffer (saponin) and stored in ice or liquid nitrogen for PCR.
- Mice inoculation is done for the highly suspected cases (presence of clinical signs).

ANIMAL SAMPLING

Clinical diagnosis

The major clinical signs include anaemia (pale mucous membranes), intermittent fever, swollen lymph nodes, gradual loss of body condition and poor appearance (dull and starry hair coat). The degree of anemia is usually the most reliable indicator of progression of trypanosomiasis disease. It is usually estimated by measuring the percentage packed cell volume of blood (PCV)

The occurrence of clinical signs may indicate that animals are suffering from trypanosomiasis but they are not associated exclusively with the disease. Thus, acute forms of trypanosomiasis may be confused with diseases such as babesiosis, anaplasmosis and theileriosis. Chronic trypanosomiasis may be difficult to distinguish clinically from malnutrition and/or severe intestinal helmithosis. A presumptive diagnosis must, therefore be confirmed by the demonstration of trypanosomes in infected animals.

- Data collection and information sheet
- Study title
- Location Date
- Name of the owner
- Animal I.D
- Species Breed Sex Age
- Body condition score
- History of treatment
- Sample collected:
- Ear vein Blood □ Venous blood □ Serum /plasma □ Lymph node aspirates
- Diagnostics Results

Wet smear	Thick smear	Thin smear	HCT	PCV	PCR	CATT/Suratex

- Remarks:
- Laboratory:
- Name of the examiner and signature:

Materials and Methods

- Ear vein blood is collected into 3 heparinised capillary tubes
- Prepare wet mount from either capillary tube blood or directly from the ear vein and examine 25 fields microscopically at 10x 40x magnification.
- Carry out HCT using 2 capillary tubes for buffy coat examination (Woo, 1970) and PCV estimation (SOPs)
- One capillary tube blood is spotted on filter paper for PCR (SOPs)
- Thick and thin smears are made directly from the ear vein blood, fixed in absolute methanol and stained in Giemsa (SOPs) for species identification
- 3-5ml of blood is drawn from the jugular vein and aliquoted for serum/plasma preparation, rodent inoculation (SOPs) and cryopreservation (Cunningham et al., 1973).
- USE OF FTA CARDS

Issues to be considered when sampling laboratory animals

Rodents

- These have to be appropriate for the disease of interest (may include criteria for selection)

- Sample size may be limited by space availability and cost of maintenance; however, the size has to be large enough to make inferences about the population
- Indicate species, breed, weight, age and source

Large animals

- Select appropriate animals for the disease of interest
- Sample size may be limited by space availability and cost of maintenance; however, the size has to be large enough to generate adequate data for inferences to be made.
- Indicate species, breed, weight, age
- Source: animals should be acquired from an area free from endemic trypanosomiasis and not exposed to trypanocidal drugs especially where the animals have to be used in drug screening
- Acclamatization: Usually takes 2-3 weeks of screening of the animals for haemo-parasites, helminths and treatment administered accordingly. The animals have to be ascertained suitable for experiments following the screening
- Housing: Ensure adequate space, fly proofed, air-conditioned where possible with adequate separation from other on-going work that could result in cross-contamination or mix-up during sampling
- Time of sample collection and frequency
- Sample storage

PARASITOLOGICAL TECHNIQUES

These techniques depend on estimation of the level of anemia and the demonstration of trypanosomes in the peripheral blood of infected animals.

Detection of trypanosome

1. Haematocrit centrifugation technique (HCT)

- Determine PCV by the haematocrit centrifugation technique
- Prepare capillary holder by fixing two pieces of glass 25 X 10 X 1.2 mm thick on a glass slide to create a 1.5 mm groove
- Place the capillary tube horizontally on the groove, place a cover slip on top and flood the gap with water or oil
- Examine the plasma/ buffy coat interphase under a microscope, focussing through the depth of the capillary tube

2. Buffy coat examination technique

- Determine PCV by the haematocrit centrifugation technique
- Cut the capillary tube 1 mm below the buffy coat and transfer the buffy coat and top layer of red blood cells on to a clean slide, mix and cover with a cover slip.
- Examine the preparation using phase contrast or dark ground miscoscopy
- To provide phase contrast, a combination of Phaco 2 NPL 25/0.50 objective, a Zernicke 402 condenser and a periplan NF x 10 eyepieces may be used (Murray et al., 1977).

3. Wet films

A wet blood smear consists of a small drop of fresh blood obtained from an ear or tail vein placed on a slide, covered with a coverslip and examined for trypanosomes. The parasites are detected by their movement or by the disturbance they cause among the blood cells. Examination of wet films provides a quick and easy means of diagnosis in the laboratory.

4. Thick films

A drop of blood is placed on a clean microscope slide and a thick film prepared by using the corner of another slide to produce a rounded smear of 1 – 2 cm in diameter. The slide is then rapidly air dried, placed in distilled water for five minutes to dehaemoglobinise prior to staining in 10% Giemsa and examination under the microscope. This technique is the most commonly used method for routine diagnosis of bovine trypanosomiasis in the field. Thick films are usually used in conjunction with thin films in which species identification can be made more confidently.

5. Thin films

A drop is placed at one end of a clean grease-free microscope slide and a thin film prepared by spreading the drop along the length of the slide with a straight edge of another slide or rectangular coverslip, quickly air-dried and fixed for three minutes in methanol. The film is then stained in 10% Giemsa for 30 minutes followed by examination for parasites under oil immersion.

6. Mouse sub-inoculation

Blood from an infected animal in 0.2 ml phosphate-buffered-saline-glucose, pH 8.0, is inoculated intraperitoneally into individual mice. Wet film preparations of tail blood from these mice are then examined three times a week for at least the following 30 days for the detection of trypanosomes.

Sampling for haematology

Haematological studies generate information on blood and diseases that affect blood composition. Thus, profiles of selected blood parameters may give an indication of occurrence, severity and type of anaemia.

Procedures in haematology

1. **Packed cell volume (PCV)**

- Puncture the ear vein using a sterile lancet and draw blood into a heparinised capillary tube
- seal one end of the tube with cristaseal or plastacene
- Place tube in the haematocrit centrifuge with sealed end outermost.
- Centrifuge at 12,000 rpm for 5 minutes
- Read the PCV in a PCV reader as percentage of the packed blood cells to the total volume of whole blood.

6. **Total RBC count, MCV, MCH, MCHC, RDW, Platelets, Mean Platelet Volume, Haemoglobin, HCT**

Under laboratory conditions where haematology analysers are available, a complete haematology profile can be determined. This involves parameters such as RBC, HB, MCV, MCH, MCHC, Platelets, MPV and RDW; also WBC and differentials.

- Collect 1-2 ml of whole blood from an appropriate vein (e.g jugular in cattle, inguinal veni-puncture in vervet monkeys)
- The blood may be collected in commercially available vacutainer tubes containing heparin or EDTA anti-coagulants. Alternately, the blood may be collected using syringe and needle and dispersed into sample bottles into which EDTA has been added to make a concentration of 1.5 mg/ml of blood.
- Analyse blood within 1-3 hours
- For the analysis of blood, follow SOPs that are specific for each analyser.

Sampling for clinical biochemistry and immunology

During monitoring of disease in both animals and humans, one may be interested in levels of specific immunological parameters whose profiles are expected to change during the course of disease. Similarly, in the generation of safety data required for drug registration, one may be interested in determining the levels of components e.g. enzyme levels before and after dosing in order to assess toxicity of a given compound.

- Collect 2-3 ml whole blood in appropriate anti-coagulant as described above
- Centrifuge the blood at 1000g for five (5) minutes (or as described in SOPs for the desired analyte)
- Separate the plasma into clean vials and store at –20°C until required for use

Sampling for histopathology

At the end of the experimental period, animals may be sacrificed for tissue examination or abnormalities, or more particularly, for changes in tissues and organs that are associated with disease; a deviation from the normal healthy state.

In carrying out post-mortem examinations, pathologists examine the animal body by system, taking samples from all the major organs for histopathological examination. The samples are fixed in 10% formalin before processing for histopathology.

VECTOR SAMPLING

Purpose

The purpose of vector sampling is to:

- Generate fundamental biological knowledge
- Develop/improve appropriate control strategies
- Provide decision support tools

Objectives

Vector sampling is geared towards determining the:

- infection rates
- vector host preferences
- species diversity
- population structure and dynamics
- vectorial competence
- vector distribution

General sampling protocol

- Carry out literature review to re-ascertain gaps in knowledge and streamline guidelines to activities
- Undertake reconnaissance visit to select the study area
- Lay out design e.g. trap density dependent on vector density and vegetation cover
- Select and georeference sites based on ecological factors that affect occurrence and distribution of the vectors such as vegetation type, altitude, water bodies, human activities, availability of hosts
- Select a suitable sampling decoy (targets, attractants)
- Use SOP for decoy deployment and harvesting (at least 100 flies)
- Identify each individual vector using morphological characteristics (Using CIRDES Species Identity Cards
- Age the flies by:
- Wing fray for males
- Wing fray and ovarian methods for females
- Depending on the objective for the survey, use the following specific SOP’s:

1. SOP for infection rate

- Dissect fresh non-tenerals at the proboscis, midgut and salivary glands
- Record the infection statues of each dissected part
- Identify the infective trypanosome at each part using the method of (Lloyd and Johnson, 1924) based on morphology formation and motility characteristics
- In case you have to do further analysis, elute all samples into ampoule (1.5 ml vials) with 70% Ethanol
- DNA extraction and analysis to be done as in the molecular techniques SOP

2. SOP for Blood meal analysis

- Empty traps every 1-2 hours to avoid digestion of ingested blood
- Select engorged flies on the basis of abdominal size and colour
- Open the upper lower abdominal sclerite by pulling gently with a sharp needle

- Extract the gut contents and smear the blood meal on a sodium azide impregnated filter paper
- Air dry for at least 30 minutes
- Store in a desiccators containing hygroscopic silicon beads for delivery to the lab
- Store at +4 ^{0}C in the refrigerator awaiting analysis

3. SOP for population genetics

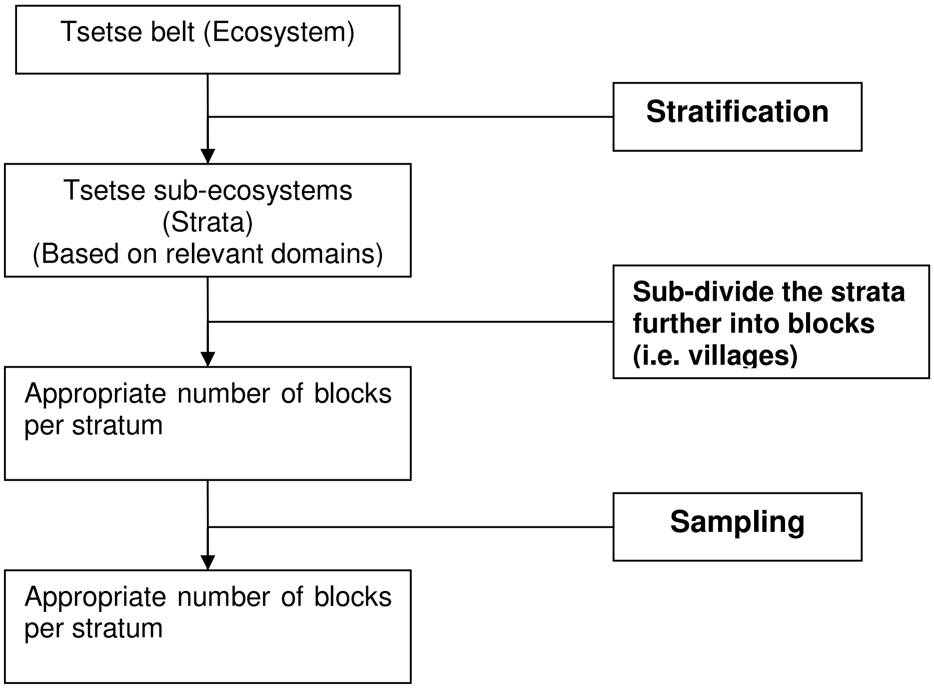
- Identify each fly using morphological characteristics to species level and sex
- Age the flies using wing fray and ovarian method if required
- Excise the legs and place them in a dry 1.5ml vial for each fly
- Provide individual identity number that should be written on the vial (e.g. M01 for fly number one from Mombasa)
- Store the samples at –20^{0}C once in the laboratory until DNA extraction is done
- Extract DNA by:
- a) Chelex ® 100 DNA extraction procedure, OR
- b) Salt extraction method
- The vial of the extracted DNA should bear the identity number of the sample
- Use dye labeled microsatellite primers for PCR
- Submit PCR products for ABI fragment analysis

4. Tsetse trapping protocol

The choice of trapping device for tsetse sampling or control is dependent on the target species. However, some traps such as biconical (Challier and Laveissiere, 1973) are known to be fairly sensitive to a number of tsetse species. In general, trapping protocol is as outlined:

- Depending on the tsetse density and the objective i.e. sampling (monitoring) or control, determine the trap density
- Having determined site intervals within transects, select trapping sites on the basis of vegetation types likely offer suitable refuges and resting sites (this requires a background on species habitat choice and behaviour)
- Georeference the trapping site using global positioning system (GPS)
- Clear an area of about 1.5 m radius for setting the trapping device. The device should be set with the entrance upwind.
- The baits e.g. olfactory attractants such as phenols, acetone or cow urine should be placed 5-10 cm from the base of the device.
- Dispensers should be capped and a hole of specific diameter for intended release rate bore at the centre. This is important in protecting the attractant from rain and also in directing the vector as the odour plume will not be dispersed
- Harvesting of trapped flies should be carried out periodically at 24 and 48 hour intervals in high and low tsetse density areas
- Trapped flies should be sorted out in to species and then by sex and age depending on the objective of the trapping

This sampling procedure is schematised below:



Remarks

Important definitions

Epidemiology has been defined as the study of the distribution and determinants of health related states or events in specified populations, and the application of this study, to the prevention and control of health problems (Last, 1988). Thus, epidemiologists are concerned not only with studying health, disease and death but also with devising means to prevent illness and improve health.

Trypanosomosis = Trypanosomiasis (HAT, nagana, Afirican Trypanosomiasis)

Protocols to be adopted in vector, animal and human sampling

- Detection of drug resistance in tsetse-transmitted trypanosomes of African domestic cattle (Eisler et al., 1999)
- ELISA for drug detection and monitoring (Murilla et al., 1999; Karanja et al., 2003; Eisler et al., 1996)
- Bioassay method for melarsoprol (Brun et al)

Bio-bank

Issues were raised on the need to establish a Bio-bank (National or Regional?)

- Feasibility studies need to be done
- Source of funding to be established
- The cost benefits of such a project to the scientific fraternity and general public (in particular African) to be identified

Protocols for sample collection could be established only after addressing the above issues