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Isolation and Cryopreservation of Trypanosomes and their Vectors for Research and Development in Resource-Constrained Settings

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Additional information is available at the end of the chapter

Abstract

Biorepositories for biological samples have increasingly become very important in supporting biomedical research since the 1990s. The Kenya Trypanosomiasis Research Institute Cryo-bank for trypanosomes and their vectors was established in the 1970s with the aim of providing research materials to scientists. Over 2000 trypanosome isolates have been collected and stored in dewars under liquid nitrogen. Recent collections include tsetse flies—vectors of human and animal trypanosomiasis. Challenges encountered include distances to remote field sites and impassable roads and the cost of collection, preparation, storage, and maintenance under resource-constrained settings. Under these settings, the challenges can be overcome through strategic leadership that ensures availability and sustainability of resources, appropriate institutional policies, adoption of multidisciplinary approach where appropriate, working with different sectors such as human health, livestock, and wildlife, and environmental conservation in order to leverage on capacities in these sectors, and acknowledging the role of communities from which materials are collected.

Keywords: cryo-bank, cryopreservation, trypanosomes, stabilates, tsetse flies

1. Introduction

Cryopreservation is an established practice of freezing and storing valuable biological materials in liquid nitrogen for long periods of time for use in research, medicine, environmental studies, and technology development. These materials include parasites, vector tissues,

and organs, a wide range of human stem cells, plants, microorganisms, etc. Research on these materials assists in understanding how ecosystems function, how disease transmission takes place, how human bodies function, and why some vectors of the same species are efficient at disease transmission whereas others are not able to transmit. Recognizing the importance of collections of biological materials to research and development and acknowledging the high cost of field sample collection in terms of financial resources and time, the management of the Kenya Trypanosomiasis Research Institute (KETRI) has put in place an institutional policy of continuous collection of trypanosome parasites for cryopreservation. This took advantage of all field visits undertaken by various scientific research teams to different foci in Kenya, a country that is endemic for both human and animal trypanosomiasis. This resulted in the establishment of the KETRI Trypanosome Bank which currently has over 2000 isolates [1] from various hosts (tsetse flies, human, domestic and wild animals). Some of the recent collections include vectors of trypanosomiasis, the tsetse flies. Updating of the cryo-bank with fresh trypanosome isolates is a continuous process.

Trypanosomes are extracellular protozoan parasites which cause debilitating disease in humans and animals. In humans, the disease is referred to as human African trypanosomiasis (HAT) or sleeping sickness, caused by two trypanosome species, *Trypanosoma brucei gambiense*, responsible for the chronic form of HAT in West and Central Africa, and *T. b. rhodesiense*, which causes acute disease in eastern and southern Africa. The parasites are transmitted by tsetse flies (*Glossina* spp.). In animals, the disease is referred to as African animal trypanosomiasis (AAT; nagana in cattle, sheep, and goats; surra in camels) and is caused by various trypanosome species, the major ones being *T. vivax*, *T. congolense* and *T. evansi* [2]. Whereas majority of the trypanosome species which cause AAT are transmitted by tsetse fly vectors, *T. evansi*, is transmitted mechanically by biting flies such as *Tabanus* spp.; *T. vivax* has been reported to be transmitted by both tsetse flies and biting flies [3].

HAT is classified in the category of the most neglected tropical diseases. Current diagnostic tools have inadequate sensitivity and specificity, thus complicating disease diagnosis and staging. The drugs available for treatment are highly toxic and not very effective; patients die if untreated [4, 5]. In 2005, an annual prevalence of 50–70,000 HAT cases/year was reported, with incidence rates of 15–17,000 cases/year [6]. Although recent data from the World Health Organization (WHO) shows that the number of reported cases of HAT declined to less than 10,000 in 2009 leading to speculation that the disease could be eliminated [7, 8], there is great need to maintain vigilance through surveillance and research. This is informed by the fact that HAT was effectively controlled in the 1960s in many endemic countries; however, the disease re-surged due to breakdown in surveillance and control activities (**Figure 1**). WHO [9] has developed a roadmap for elimination of HAT by the year 2020, which involves development of new and better diagnostics and drugs [5, 8]. Cryo-banks such as the KETRI Trypanosome Bank will therefore be important in contributing to this strategy in order to ensure that epidemics do not occur in future; and that dormant foci will be prioritized for elimination. One of the issues for which answers are sought is what happens in some traditional HAT foci when the disease is not reported in humans. Some of the new technological advances that are providing more insights include genetic analysis of both parasite and vector genomes and

identification of specific proteins as targets for development of vaccines, new and sensitive diagnostic tests.
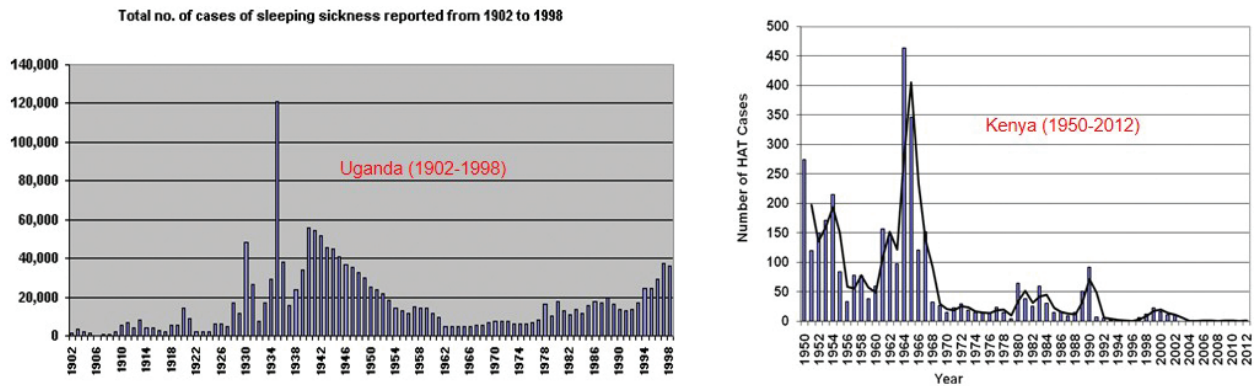

Figure 1. Sleeping sickness as a reemerging disease.

Isolation and cryopreservation of new trypanosome strains from patients in different HAT foci ensures availability of these stabilates for use in parasitological, biochemical, molecular, serological and pharmacological investigations many years after their isolation from the host. Brun *et al* [2] observed that one of the major obstacles in the elucidation of the factors responsible for relapses after melarsoprol treatment was the lack of recent *T. b. gambiense* isolates from patients from various endemic areas where the problem has been reported. The WHO steering committee on human African trypanosomiasis treatment has therefore recommended that collection of stabilates be a continuous activity in order to monitor the occurrence and spatial distribution of treatment failure [10] and refractoriness of tsetse to infection. Since its inception, KETRI and now KALRO-Biotechnology Research Institute developed an institutional policy of encouraging collection of stabilates by scientists and clinicians, for cryopreservation. In this chapter, we describe the procedures of isolation and cryopreservation of trypanosome stabilates for research and development in resource constrained settings.

2. Field isolation of trypanosomes

Trypanosomes are isolated from infected hosts during active or passive disease surveillance activities. The infected hosts include humans, domestic and wild animals, as well as tsetse fly vectors. Parasites are isolated from biological fluids including blood, cerebrospinal fluid (CSF) and lymph node aspirates, and/or body parts of tsetse fly vectors. Depending on the host parasitemia and/or density of trypanosomes in the biological fluids at the time of isolation, trypanosomes can be either cryopreserved directly or propagated in immunosuppressed laboratory rodents prior to cryopreservation.

2.1. Diagnosis: buffy coat and whole blood parasitemia

Parasitological diagnosis of trypanosome infections in animals and humans can be made through microscopic examination of wet blood smears, stained thin and thick blood smears, smears of lymph node aspirates, and buffy coats [11]. Under normal field conditions when large numbers of animals are sampled, examination of buffy coats, obtained through capillary tube centrifugation technique (CTC) [12], is the preferred method of diagnosis due to its higher sensitivity compared to other microscopic techniques. Animals suspected to be infected with trypanosomes are bled from the ear vein into heparinized capillary tubes after which the 3/4 full capillary tubes are sealed at one end with plasticine and then spun in a hematocrit centrifuge at 10,000 revolutions per minute for 5 min. Blood separates into three portions, namely, the red blood cells, which settle at the bottom of the capillary, the plasma portion found at the top, and the buffy coat portion, which forms at the interface of the red blood cells and plasma. Trypanosomes are concentrated in the buffy coat portion of the centrifuged blood, thus enhancing the sensitivity of the test. In humans, diagnostic methods that are routinely employed to detect blood trypanosomes include (CTC), quantitative buffy coat (QBC), mini anion exchange centrifugation technique (mAECT), and modified mAECT [13]. For the diagnosis of trypanosomes in cerebrospinal fluid, available methods include single and double centrifugation and modified single centrifugation (MSC), with the MSC being easy to perform and as sensitive as the double centrifugation [13].

Once confirmed positive, the density of trypanosomes in the relevant biological, fluid is determined. Whole blood is drawn from the jugular vein of the infected host into anticoagulant containing tubes and used to quantify the parasitemia using the matching method [14] for the Trypanozoon group of trypanosomes. Direct isolation is therefore determined by whole blood parasitaemia.

2.2. Direct isolation of parasites from infected biological fluids

Parasitemia is usually low in naturally infected hosts. However, the required density of between 3.2×10^7 trypanosomes/ml and 1.3×10^8 trypanosomes/ml may be obtained in a small proportion of the infected hosts, thus permitting direct cryopreservation of the stabilates. In such cases, the infected whole blood is mixed with either of the following cryoprotectants and processed:

1.   20% glycerol in EDTA saline glucose (ESG), pH 8.0 in the ratio of 1:1.

2.   Glycerol in the ratio of 1:4, that is, one part of the infected blood to four parts of glycerol.

Samples are then labeled and dipped into a vapor shipper liquid nitrogen cylinder for transportation from the collection site to the main laboratory for further processing. A vapor shipper is a liquid nitrogen cylinder which has a mechanism of absorbing liquid nitrogen into its system leaving the hollow space full of liquid nitrogen vapor, with temperature in the range of −60 to −80°C. In the laboratory, samples are removed from the vapor shipper and allowed to thaw on ice or at 4°C after which they are dispensed into plain capillary tubes (**Figure 2**), which are then sealed at one end using plasticine.
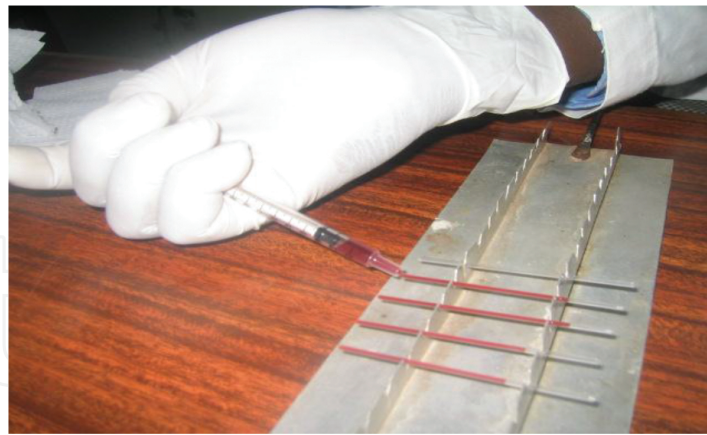
Figure 2. Loading plain capillary tubes with cryo-protected infected blood sample.

Top holds one sample

Top of the cane: both the cane and canister numbers are written on top of the cane as shown by the arrow, held in position with adhesive tape; starting with cane followed by canister number.
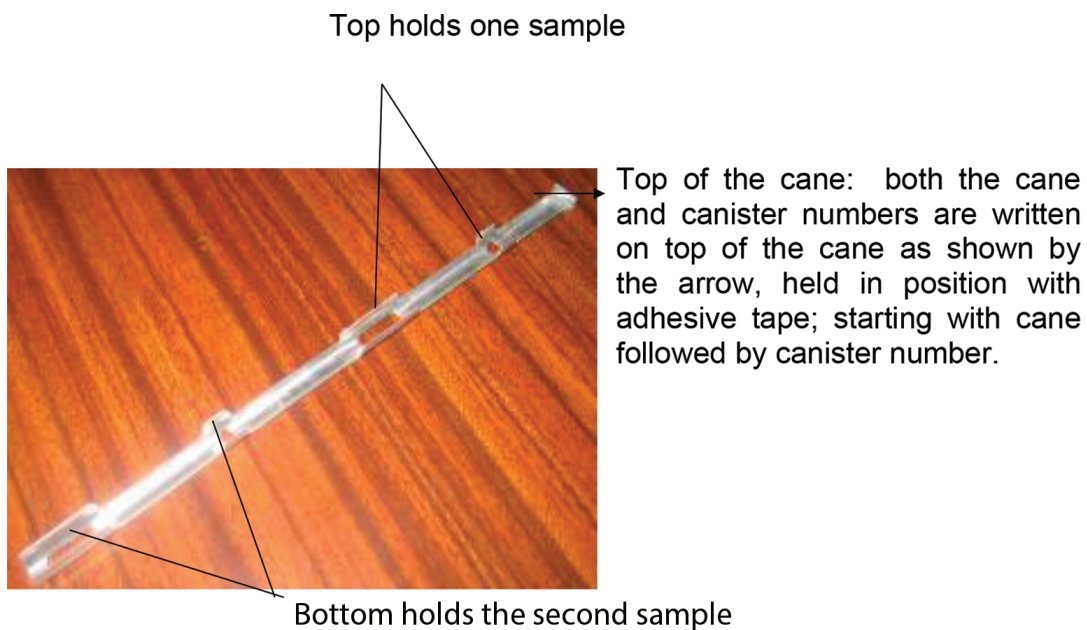
Bottom holds the second sample

Figure 3. Aluminum cane for holding the ampoules.

The loaded and sealed capillary tubes, approximately 18 in number, are then accommodated in a perforated 4.5 ml ampoule tube into which a label is inserted. The label has the laboratory sample identification number. The ampoule normally has two perforations, one at the top and the other at the bottom to allow direct contact of the sample with liquid nitrogen and at the same time continuous flow of liquid nitrogen from the top through the bottom perforations. Before permanent storage, a capillary is removed from the suspended sample and the viability of the trypanosomes confirmed. For ease of permanent storage in liquid nitrogen at –196°C, the ampoule is then placed on to an aluminum cane which has the capacity of holding at least two ampoules: one at the top and the other at the bottom of the cane. The cane is then placed into a canister, with each canister having a capacity of holding 14 canes. The canister is then immersed into liquid nitrogen in the permanent storage dewar (**Figures 3** and **4**) at –196°C.

Figure 4. Stabilate storage dewars.

2.3. Isolation and propagation of low parasitemia blood samples in laboratory rodents

When the parasitaemia in the whole blood of the naturally infected host is low i.e. below 1.3×10^8 trypanosomes/ml, the anticoagulated infected blood sample is intraperitoneally inoculated into an immunosuppressed laboratory rodent. It is advisable to inoculate two rodents, usually mice, per positive blood sample (isolate). This is carried out in the field where the animals are given identification numbers and transported to the laboratory for monitoring. In the laboratory, the infected mice are maintained on commercial mice pellets (Unga Feeds Ltd., Kenya), provided with water *ad libitum* and monitored for development of parasitemia. At the first peak of parasitemia between 1.3×10^8 trypanosomes/ml and 2.5×10^8 trypanosomes/ ml, the infected mice are euthanized and the blood harvested by cardiac puncture into tubes containing EDTA as anticoagulant. The blood is mixed gently before addition of a cryopreservative at a ratio of 1:1. The samples are suspended in liquid nitrogen vapor for at least 2 h using a cooling jacket before permanent storage in liquid nitrogen at –196°C [15, 16]. Samples with a trypanosome concentration below 6.3×10^7/ml may also be preserved by direct addition of glycerol to infected biological fluids to a final concentration of 20%, especially if the isolated trypanosome species does not infect rodents [17].

3. Cryopreservation

3.1. Cryopreservation media

The most commonly used cryopreservative is 20% glycerol in EDTA saline glucose (ESG). ESG is first prepared by dissolving 8.00 g NaCl, 0.30 g KH_2PO_4, 2 g EDTA (disodium or di-potassium), and 2 g glucose in 800 ml distilled water, adjusting the pH to 8.0, and then topping up to 1 liter with deionized/distilled water. Glycerol is then added to the prepared solution at a ratio of 1:4 to make 20% glycerol in ESG. Recently, Triladyl® (MiniTüB GmbH and CO. KG, Tiefenbach, Germany), a commercially available culture medium that is traditionally used for preserving bull semen, has been found suitable for cryopreservation of trypanosomes. Triladyl is most efficient when added to the infected biological fluids/materials

to a concentration of 40–90% [18, 19]. Triladyl has subsequently been adopted as an alternative cryopreservative at the KETRI Trypanosome Bank.

Buffers commonly used in cryopreservation

EDTA saline glucose (ESG) pH 8.0

NaCl 8.00 g

KH_2PO_4 0.30 g

EDTA (disodium or dipotassium) 2 g

Glucose 2 g

Dissolve in about 800 ml distilled water, top up to 1 l.

Normal saline

Dissolve 8.5 g NaCl in 1 l of distilled water

Phosphate Saline glucose (PSG) pH 8.0

Na_2PHO_4 5.392 g/l

$Na_2HPO_4 (H_2O)_{12}$ (hydrous) 13.608 g/l

NaH_2PO_4 0.239 g/l

$NaH_2PO_4(H_2O)$ hydrous 0.276 g/l

$NaH_2PO_4(H_2O)_2$ hydrous 0.312 g/l

NaCl 1.7 g/l

Glucose 10.00 g

Dissolve in about 800 ml distilled water; top up to 1 l.

3.2. Cryopreservation procedure

Biological materials from infected vertebrate hosts (blood, cerebrospinal fluid (CSF), and lymph node aspirates) and/or body parts of tsetse fly vectors are processed as previously described [17, 20, 21]. Briefly, samples with a concentration of more than 6.3×10^7 trypanosomes/ml are mixed with a cryopreservative (20% glycerol in ESG or 40–90% Triladyl) at a ratio of 1:1. The diluted sample is then loaded into an ampoule and wrapped or held in a cooling

jacket which is suspended into the vapor space of a liquid nitrogen shipper (temperature range of –60 to –80°C) for transportation to the laboratory. Samples from infected humans or animals with parasite counts below 6.3×10^7 trypanosomes/ml (equivalent to antilog 7.8) [14], or suspects with a low packed cell volume (usually <25%) are inoculated into laboratory rodents for amplification/propagation [19]. The rodents may be immunosuppressed using either cyclophosphamide at 100 mg/kg daily for three consecutive days or by gamma-irradiation at 600 rads (6 gray) for 6 min before the inoculation [22, 15]. Swiss white mice are preferred for propagation of most species of trypanosomes, while Mastomys natalensis are preferred for *T. b. gambiense* [23]. Inoculated rodents are sacrificed at the first peak of parasitemia and the blood harvested by cardiac puncture into tubes with EDTA as anticoagulant. The blood is mixed gently before addition of a cryopreservative at a ratio of 1:1. The diluted samples are then suspended in liquid nitrogen vapor for at least 2 h before being stored permanently in liquid nitrogen at –196°C [24, 25].

Essential requirements for cryopreservation

1.   Immunosuppressed mice

2.   Mice pellets

3.   Sawdust

4.   Disposable 1 ml syringes and needles gauge 26

5.   Trypanosomes either from the cryo-bank or from infected hosts (humans/animals) or tsetse flies

6.   Glycerol

7.   Cotton wool

8.   Tissue paper

9.   Capillary tubes (plain)

10.   Plasticine

11.   4.5 ml plastic ampoules

12.   Liquid nitrogen

13.   Cooling jacket

The following information is collected and recorded:

1.   Host of isolation

2.   Locality (georeferenced) and year of isolation

3.   Scientist who did the isolation

4.   Number of passages the trypanosomes has undergone before cryopreservation

5. The prepatent period of the infected mice after each passage, if several passages have been done

6. The duration of infection in the infected mice between the time of inoculation and sacrifice of the mouse for cryopreservation

7. Species of trypanosome

8. Physical location of the sample in the cryo-bank

In event of cryopreservation from the natural host:

• The suspected infected blood sample is injected into immunosuppressed mice

• The infected mice are monitored for development of parasitemia

• Cryopreservation carried out as outlined above

At the KETRI trypanosome cryo-bank, the first parasite population isolated from the field is the original or primary isolate. If the parasite numbers are high, the primary isolate is cryopreserved as original field isolate; however, in order to sustain/maintain the original cryopreserved sample and to produce adequate material for research, the original isolate is expanded in the appropriate animal model and cryopreserved as a derivative of the original sample, but with a different bank reference number from the first passage number. Subsequent passages or derivatives of the same are given different reference numbers in order to monitor the use and performance of the particular isolates. Clones may be prepared either from the primary or subsequent passages or both. All these events are monitored and recorded for future reference and when issuing materials for research.

3.3. Preparation of the cryopreserved sample for the infection of laboratory animals

Following receipt of the duly signed and approved request form from the scientist, the person incharge of issuance of cryopreserved material records the physical position of the sample in the cryo-bank. The sample is retrieved from its position, the ampoule cork opened; and using a pair of forceps, the reference number is confirmed. One capillary is removed and transferred into an ampoule placed on ice for thawing. The remaining stabilate is returned into its position in the bank before it thaws.

The procedure for infection of laboratory animals

1. Use 1 ml syringe and 26-gauge needle

2. Fill the syringe with phosphate saline glucose (PSG) pH 8.0 up to 0.2 ml

3. Using a diamond pencil cut the sealed end of the capillary tube containing the trypanosome stabilate

4. Insert the needle into the capillary and suck the contents

5.   Pull the piston and mix the contents with the PSG buffer thoroughly

6.   Remove the air bubbles and place a drop of the mixture on the microscope slide, cover with a cover slip, and examine the parasitemia at 400 × magnification

7.   Infect the experimental (donors) as required by the protocol

Precaution:

1.   Use of protective devices while handling cryopreserved samples is mandatory. This is necessary because the samples being handled contain live parasites, some of which are pathogenic to humans. Also, nitrogen at –196°C burns. Industrial gloves are recommended while handling liquid nitrogen. Use of facial masks will protect the user from harmful effect in case of contact with the eyes. All safety precautions should be strictly observed when capillary tubes are withdrawn from the liquid nitrogen; they sometimes burst before they are transferred into the screw capped ampoules to thaw, possibly due to differences in temperature.

2.   It is recommended that retrieval of the sample should be rapid to avoid the thawing of the remaining samples.

4. Cloning of trypanosomes

Cloning of trypanosomes is necessary for the production of a homogeneous population of trypanosomes. It is carried out as previously described by Otieno and Darji [23]. Briefly, a sample containing trypanosomes is diluted in PSG pH 8.0, to 1 trypanosome per micro-scopic field at 400× magnification. This is followed by addition of 0.5 ml guinea pig se-rum. Using a needle, a drop of the trypanosome suspension is placed on a cover slip that is overturned onto a cavity slide moistened with PSG to prevent evaporation of the drop. In the laboratory, the drop is examined under a microscope (400× magnification) by at least three experienced technicians to confirm the presence of a single and viable trypano-some, which is aspirated and inoculated into an immunosuppressed Swiss white mouse. Inoculated mice are monitored for parasitemia development and sacrificed at the first peak of parasitemia. Blood is harvested by cardiac puncture for cryopreservation of the clone of trypanosomes.

5. Maintenance of cryopreserved trypanosomes

5.1. Liquid nitrogen

Cryopreserved trypanosomes are permanently stored in liquid nitrogen. The samples must always be fully immersed in liquid nitrogen and the levels maintained by frequently refilling the storage Dewars. The refilling period is determined by the frequency at which the Dewars

are opened during issue of materials for research. The more frequent they are opened, the more liquid nitrogen vapor is lost, hence the need to refill. Under normal circumstances, refilling is done fortnightly.

5.2. Trypanosomes viability and infectivity tests

It is important to ascertain that the cryopreserved samples remain viable by randomly testing the infectivity of the parasites in laboratory rodents to ensure that this has been maintained and not lost over long periods of storage. The viability testing is performed by removing a single capillary of each of the cryopreserved trypanosome isolate, thawing at room temperature, cutting the sealed end of the capillary tube using a diamond pencil, decanting the capillary contents on a microscope slide, covering the content with a glass coverslips, and examining for the motility of the trypanosomes under the microscope at 400× magnification.

6. Morphological characterization of trypanosomes

Morphological features assist in the preliminary identification of trypanosomes in the field after isolation in order to ascertain the species of trypanosomes isolated. This is done through microscopic examination. Different trypanosome species fall into the following groups, depending on the morphological features: Trypanozoon, Duttonella, or Nannomonas (**Table 1**).

Species	Morphology	Free flagellum	Undulating membrane	Kinetoplast	Other characteristics
T. vivax	Monomorphic	Present	Slightly developed	Large rounded terminal	Very motile, posterior end rounded
T. uniforme	Monomorphic	Present	Slightly developed	Large rounded terminal	Very motile, posterior end rounded
T. congolense	Pleomorphic	Absent	Slightly developed	Marginal or central subterminal	Posterior end rounded or flat
T. simae	Pleomorphic	Absent	Moderately developed	Marginal or central subterminal	More long forms than short forms
T. brucei	Pleomorphic	Present in long and intermediate forms, absent in short forms	Well developed	Small subterminal	Posterior end: Long forms—pointed Short forms—rounded Intermediate forms—blunt
T. evansi	Basically monomorphic	Present	Well developed	Small subterminal	Posterior end rounded or truncated
T. suis	Monomorphic	Present	Well developed	Small, subterminal, marginal	Only infects suids
T. theileri	Monomorphic	Present	Well developed	Large, rounded, marginal	Posterior end tapering

Table 1. Morphological characteristics of trypanosomes.

6.1. Some of the trypanosome species distinguishing morphological characteristics include:

- Size and shape of the body

- Position of the nucleus and kinetoplast

- Presence or absence of free flagellum

- The shape of the posterior end which is pointed either sharply, oval, or blunt

The morphological features of the Trypanozoon, Duttonella, and Nannomonas species of trypanosomes are as shown in **Figures 5–7**.
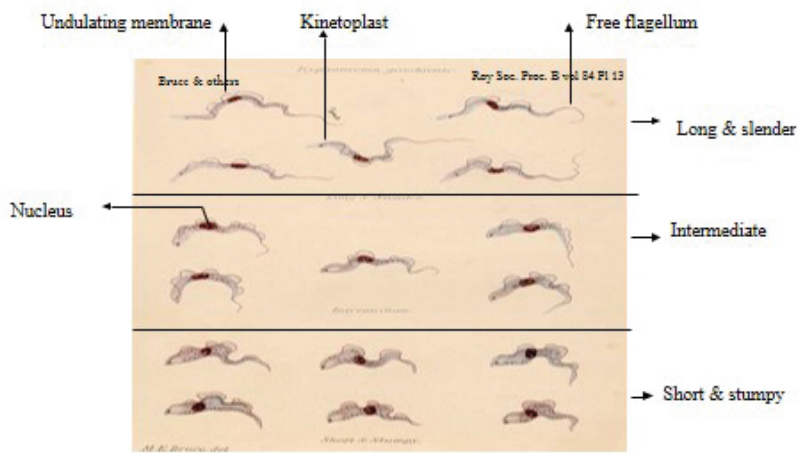

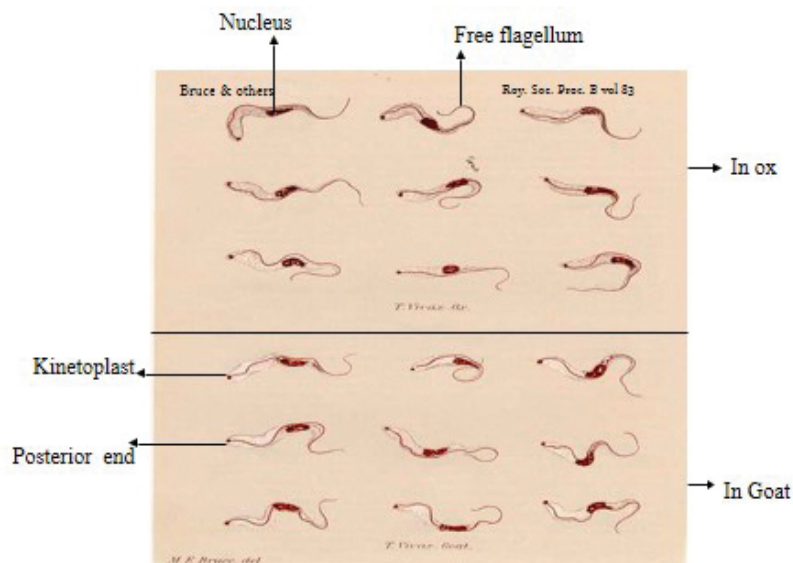**Figure 5.** Morphological distinguishing features associated with Trypanozoon.

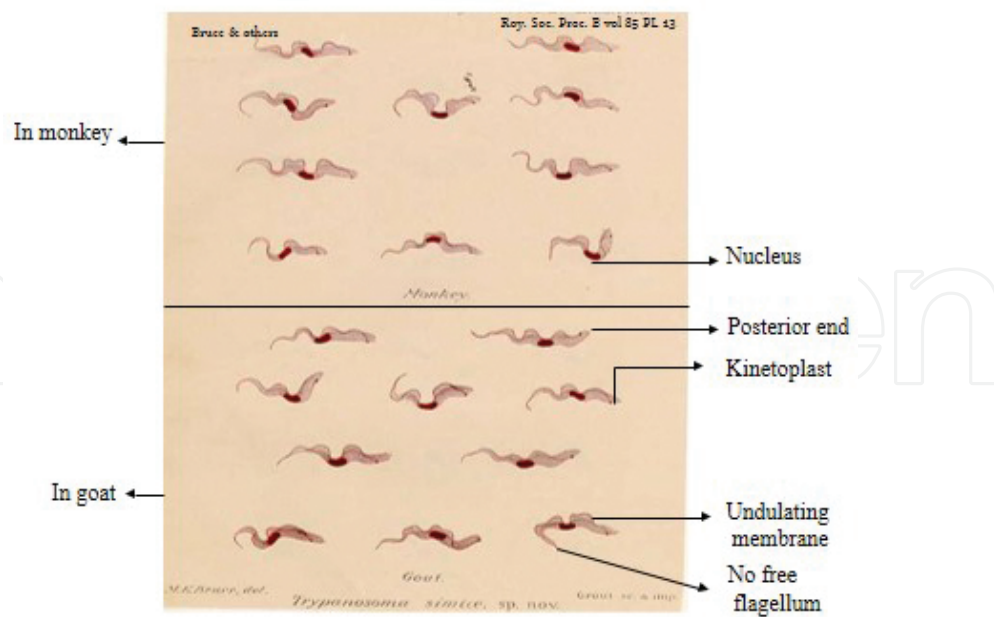**Figure 6.** Morphological distinguishing features associated with Duttonella.

Figure 7. Morphological distinguishing features associated with Nannomonas.

6.1.1. Trypanosoma brucei gambiense (Trypanozoon) subgroup

Morphological distinguishing features associated with Trypanozoon include:

- Position of kinetoplast—subterminal

- Size of kinetoplast—small

- Posterior end—blunt

- Free flagellum—present

6.1.2. Trypanosoma vivax (Duttonella) subgroup

Distinguishing features include: rounded posterior end, free flagellum, and a large almost terminal kinetoplast. This group of parasites lack undulating membrane.

6.1.3. Trypanosoma simiae in the same group with T. congolense (Nannomonas)

Distinguishing features include: shape of posterior end—rounded; position of kinetoplast —marginal; size of kinetoplast—medium; no undulating membrane and free flagellum—present.

6.1.4. Other methods employed in the laboratory to characterize trypanosomes

- Tsetse transmission studies

  Trypanosoma evansi is not transmitted by *Glossina* but by biting flies.

- Tissue culture

  Trypanosoma evansi does not transform into procyclics in culture.

- Molecular biology studies:

  ◦ DNA and RNA extraction and analysis

  ◦ Genetics and functional genomic studies

- *In vivo* studies using various animals models to characterize virulent phenotypes and drug resistance. It is important to note that some trypanosome species such as *Trypanosoma vivax* do not grow in rodents.

7. Documentation

Over the years of existence of the KETRI Cryo-bank, the cryopreserved materials were documented manually on specially designed record sheets known as Kalamazoo and later electronically. Whichever method is used, the information in the records should include, but not limited to the following, for the ease of retrieval:

- The host of isolation, age and sex

- Date of isolation

- Isolating scientist

- Suspected species of trypanosome (based on host of isolation and trypanosome morphology)

- Locality of isolation

- Method of cryopreservation whether direct or after propagation in laboratory animals

- If by propagation in laboratory rodents, the species of rodent used

- The prepatent period, i.e., period between inoculation of the whole blood and first appearance of trypanosomes in the mouse

- Duration of infection before cryopreservation, i.e., the period between inoculation and the sacrifice of the animal to harvest trypanosomes for cryopreservation

- In the event where several passages have been made, the passage numbers must be indicated as well as the species of rodents used at every passage. In addition, the pre-patent period and duration of infection must be stated at each passage.

- Physical location of the stabilate in the cryo-bank

- Work carried out and publications resulting from the use of stabilates

7.1. Electronic database

The database was developed using Microsoft Access 2000 (Microsoft, USA) relational database. Hosts of isolation and countries are coded following the International Organization for Standardization (ISO) protocol [26] (Lumsden, 1978). Primary isolates are, for example, designated MHOM/KE/85/KETRI 128, where M represents mammal; HOM represents human; KE indicates the country of isolation, in this case Kenya; 85 is the year of isolation (1985) while KETRI 128 shows code or reference number of the stabilate. With regard to trypanosome derived from the previous stabilate usually referred to as derivatives, the number of the derivative is shown in brackets. For example, MHOM/KE/85/KETRI 128 [KETRI 300] is a derivative of MHOM/KE/85/KETRI 128 as described by Lumsden [25].

7.2. Trypanosomes isolated, documented, and cryopreserved at KETRI Cryo-bank

The number of trypanosomes isolated and cryopreserved at the KETRI Trypanosome Bank are shown in **Figure 8**. **Tables 2** and **3** show the trypanosome species, year, and country of isolation.
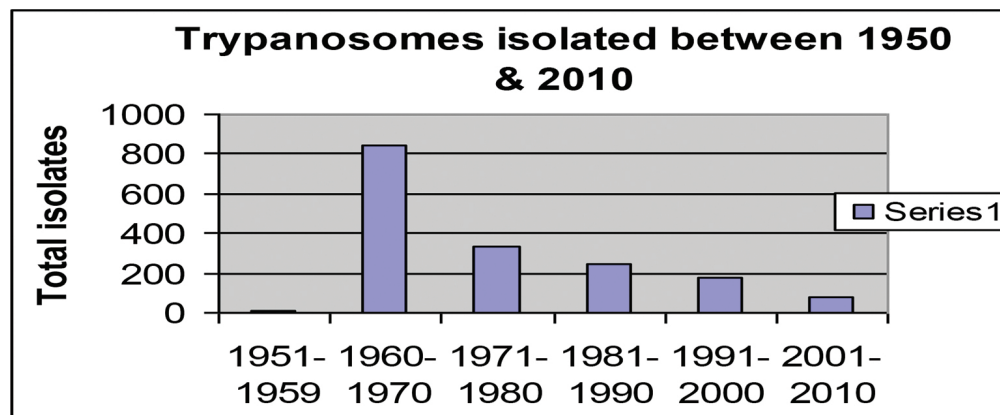

Figure 8. Number of primary trypanosome stabilates collected, preserved, and stored at Kenya [1].

			Species of trypanosomes: number and period of isolation										
Country	Isolate/ Year	Tbb	Tb subgroup	Tbr	Tbg	T. congolense	T. vivax	T. evansi	T. simiae	T. theileri	T. lewesi	UC	Mixed
Kenya	No	101	194	274	–	107	166	89	3	–	–	–	29
	Year	1961–2001	1961–2006	1958–2009	–	1961–2008	1969–2009	1968–2003	1970	–	–	18	1970–2006
Uganda	No	1	238	123	22	82	64	–	–	2	8	14	5
	Year	1968	1960–1983	1959–2004	1959–2002	1955–1983	1961–1972	–	–	1972–1973	1966	14	1955–1971
Tanzania	No	–	57	7	–	35	–	–	–	–	–	–	9
	Year	–	1966–1974	1934, 1959–1974	–	1966–1974	–	–	–	–	–	–	1966–1974
Botswana	No	–	–	2	–	–	–	–	–	–	–	–	–
	Year	–	–	1960	–	–	–	–	–	–	–		–
Sudan	No	–	–	–	26	–	–	2	–	–	–	–	–
	Year	–	–	–	1982–2003	–	–	1973	–	–	–	–	–

Country	Isolate/Year	Species of trypanosomes: number and period of isolation											
		Tbb	*Tb subgroup*	*Tbr*	*Tbg*	*T. congolense*	*T. vivax*	*T. evansi*	*T. simiae*	*T. theileri*	*T. lewesi*	UC	Mixed
Mozambique	No	–	–	2	–	–	–	–	–	–	–	–	–
	Year	–	–	1980, 1983	–	–	–	–	–	–	–	–	–
Nigeria	No	–	–	–	–	–	4	–	–	–	–	–	–
	Year	–	–	–	–	–	1970–1973	–	–	–	–	–	–
Zambia	No	–	–	–	–	2	–	–	–	–	–	–	–
	Year	–	–	–	–	1981	–	–	–	–	–	–	–
NDA	No	2	21	8	–	17	5	1	–	–	–	3	2
	Year	1961	–	–	–	1962–1985	1961	–	–	–	–	–	2
Total		104	510	416	48	243	240	92	3	2	8	35	45

Adapted from Murilla et al. [1].

Tbb, Trypanosoma bruceibrucei; Tbr, Trypanosoma bruceirhodesiense; Tbg, Trypanosoma bruceigambiense; UN, unclassified; NDA, no data available.

Table 2. Primary trypanosome isolates collected from various countries and stored at the Kenya Trypanosomiasis Research Institute Cryo-bank

	Tbb	*Tb subgroup*	*Tbr*	*T. congolense*	*T. vivax*	*T. evansi*	*T. theileri*	*T. simiae*	*T. lewesi*	UC	Mixed	Total
Cattle	85	247	0	119	137	0	2		0	10	21	621
Goat	0	6	0	6	6	0	0	0	0	0	1	19
Sheep	1	8	1	24	3	0	0	0	0	0	7	44
Pig	1	5	0	0	0	0	0	0	0	0	0	6
Camel	0	3	0	1	2	92	0	0	0	0	0	98
Donkey	0	1	0	1	0	0	0	0	0	3	0	5
Cat	0	0	0	0	0	0	0	0	0	0	2	2
Dog	3	6	0	1	0	0	0	0	0	1	0	11
Wildlife	2	40	3	12	0	0	0	0	0	0	5	62
Lizard	0	0	0	0	0	0	0	0	0	3	0	3
Rat	0	0	0	0	0	0	0	0	8	0	0	8
HNI	1	7	4	2	2	0	0	0	0	2	0	18
Total	93	323	8	166	150	92	2	0	8	19	36	897

Adapted from Murilla et al. [1].

Table 3. Animal hosts from which various trypanosomes were isolated and stored at Kenya Trypanosomiasis Research Institute Cryo-bank

8. Issuance of stabilates

Trypanosomes are stored and maintained permanently in liquid nitrogen for use by scientists in the following areas:

1. The pathogenicity/virulence studies

2. Molecular characterization

3. Drug sensitivity studies

4. Tsetse fly transmission/vector competence studies, etc.

5. Genetic and genomic studies

6. Research and development of new diagnostic tests, drugs, and vaccines

Stabilates are issued to National and International Research Organizations and Institutions of higher learning following the laid down institutional guidelines. The following documents are necessary for issue of materials to be effected:

Issuance of trypanosome stabilates

Procedure

1. Research permit from National Council for Science, Technology and Innovation (NACOSTI, Kenya)

2. Stabilate requisition form plus a permit from Director of Veterinary Services (DVS) for stabilates to be used in other National and International institutions

3. Materials transfer agreement duly signed

4. Stabilate requisition form with relevant approvals for stabilates to be used only within the institute.

It is important that the receiving scientist be committed to avail the scientific information resulting from the use of these stabilates. This is necessary for updating the trypanosome bank database and for future reference.

9. Terminologies commonly associated with cryopreservation (from: Trypanosomiasis a veterinary perspective Lorne E. Stephen pages 440–441)

Terminologies commonly used with cryopreservation technique

1. Trypanosome species

   Assemblages of organisms that can be distinguished from other species by one or more stable discontinuous morphological characters, e.g., *T. congolense*, *T. vivax*, *T. brucei* are different species.

2. Trypanosome subspecies

   Assemblages of organisms within a species that cannot be separated from each other by morphological characters but only by other stable characters, e.g., *T. b. rhodesiense*, *T. b. gambiense* are subspecies of *T. brucei*.

3.   **Clone**

This is a population that has been developed from a single trypanosome.

4.   **Line**

A laboratory derivative of a stock maintained in different physical conditions, e.g., a species of *T. congolense* maintained in mice only is a line of that nature, while when maintained in cattle only is another line of that nature, etc.

5.   **Population**

The group of trypanosomes present at a given time in a given host or it may consist of a mixture of several species and subspecies.

6.   **Primary isolate**

This is a stabilate made from a naturally infected host or viable organisms present in a culture or experimental animals following the introduction of the sample from a naturally infected host.

7.   **Sample**

That part of trypanosome population collected on a unique occasion.

8.   **Stabilate**

A cryopreserved sample of viable trypanosomes.

9.   **Stock**

A population derived by serial passage *in vivo* or *in vitro* from a primary isolate.

10.   **Metacyclics**

These are the mammalian infective forms of trypanosomes injected by the tsetse fly. Metacyclic forms are found at the end of transmission cycle.

11.   **Procyclics**

These are the midgut forms of trypanosomes which are found in cultures.

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10. Conclusion

Communicable and noncommunicable diseases, including the neglected tropical diseases, cause chronic life-long disability, hinder economic development, and impair childhood development in resource poor settings in Africa where the diseases are endemic [27, 28]. Control of these diseases could be an efficient way to fight poverty since some of these diseases can be managed very cost-effectively using evidence-based control strategies [26, 29, 30]. HAT is classified as one of the most neglected tropical diseases that exclusively affects poor communities in low- and middle-income countries (LMIC), except those areas where tourists

have been reported to have contracted the disease on tour of the affected areas. Because NTDs affect mostly the socially vulnerable populations, there are several ethical implications to consider when planning collection and use of these materials. Detection and treatment of these diseases poses many challenges since most of them present similar clinical symptoms concomitant with variation in response in the affected individual to treatment, and lack of accurate diagnostic tests. Medical research to improve health care faces a major problem in the relatively limited availability of adequately annotated and collected bio-specimens, primarily due to absence of bio-banking facilities and associated infrastructure to interrogate the specimen to tease out relevant information. This limitation has adversely impacted the pace of scientific advances and successful exploitation of bio-specimens. Established functional bio-banks would surmount this limitation by providing framework for transfer of bio-specimens (tissues, blood, and body fluids) and related health data for research. The KETRI Cryo-bank holds significant quantities of samples dating from 1930s to date, which include blood, serum, CSF, tissues, semen from trypanotolerant animals, and both parasite and vector DNA collections. This is in addition to the pan African trypanosome isolates of specific biomedical interest (e.g., drug resistance and virulence) from human and nonhuman primates, and livestock. There is therefore great need to collect and store biological materials for research in order to monitor our ecosystems including new and emerging diseases, generate evidence to inform policy, and in the development of mitigation strategies. In the area of human and veterinary medicine, these new and reemerging diseases and conditions have complicated the search for new remedies for their management in the absence of well collected and cryopreserved biological specimens.

10.1. The challenges

The countries that are heavy burdened by disease also experience high levels of poverty. This situation is compounded by new and reemerging diseases and conditions. Climate change has not only resulted in loss of biodiversity but enabled vectors to infest new areas and change transmission dynamics. Some parasites have changed host seeking behavior with time, becoming either more virulent or chronic in nature. Development of drug resistance and appearance of virulent phenotypes is of great public health concern. Whereas the need exists to collect and preserve these materials for R&D in order to find solutions to these challenges, the cost of sample collection from the field is prohibitive. Sites are usually remote with unpassable roads especially in rainy season when disease transmission is high. Once the materials have been collected and transported to the laboratory, there are high costs related to processing, cryopreservation, and maintenance of the cryo-bank. These are not the usual areas for investment by our governments due to different priorities. There are also challenges related to communities from which samples are collected, they are usually not involved in the plans to collect the materials thereby excluding them in finding solutions to their problems.

10.2. The opportunities

The above challenges have created great opportunities for collection and storage of parasites and their vectors for use in the development of vaccines, diagnostic tests, and new medicines

applying recent technological advances. In the recent past, improvements have been made on the conventional nitrogen freezers through development and adoption of validated methods including a wide range of stem cells. Many cryopreservation protocols exist for freezing and storing various biological materials. These need to be reviewed and tailored toward delivering quality biological materials to our research institutions and products to our clinics. Modalities for sharing of materials by different institutions need to be developed and made operational in order not to disadvantage communities from which the materials are collected and the institutions that have collected, preserved, and maintained these materials in resource-constrained settings. The contractual arrangements surrounding areas of the material transfer agreements should be carefully negotiated. National and international institutions (local and foreign), should invest in adequate bio-specimen management, legal and administrative skills, just as they do for developing scientific skills to facilitate sharing of samples and information associated with the bio-specimens.

10.3. Lessons learnt: how to establish and sustain cryobank in a resource-constrained setting

Collection and cryopreservation of biological materials is critical to research and development but expensive to collect, process, store, and maintain. Institutional top management leadership supported by the existing national, regional and international guidelines, rules and regulations are necessary in providing policy direction and resources [31]. In Kenya, tsetse flies, vectors of human and animal trypanosomiasis, infest mainly conservation areas and wildlife are carriers of pathogens, hence there is a need to work closely with the Kenya Wildlife Service. Through effective collaborations and multidisciplinary approach, it is possible to leverage on all activities undertaken by collaborating institutions to make collections. From the resource-constrained perspective, one does not need state-of-the-art bio-repository to initiate collections. Strategic leadership is the key in spearheading:

1. the development of the appropriate institutional policies

    1. to define roles and responsibilities for collaborative arrangements among institutions, strictly observing existing rules and regulations

    2. to ensure that communities from which the materials are collected are not taken advantage of

    3. to facilitate the establishment of relevant multidisciplinary teams that cut across several sectors, e.g., human health, livestock, and wildlife

    4. to ensure proper collection, storage, and maintenance of the materials according to the legal mandates of participating institutions

    5. to ensure equity in sharing of the resources,

2. capacity assessment and development to include human, infrastructure, and financial across the sectors, and

3. training of field teams in best practices regarding sample collection and processing at field level [32–34].

Effective coordination of field teams is critical as many of the areas from where the collections are made are remote with no electricity. Due to the rough terrain and impassable roads, especially during the wet season and when the disease transmission is high, a lot of liquid nitrogen may be lost. In view of this, it is important to ensure adequate liquid nitrogen is available to last the period of the field trip. This is critical and assures viability of the parasites from the remote field sites to the laboratories for preparation and permanent storage.

In conclusion, it is possible for institutions to collect, process, store, and maintain biological resources according to their legal mandates in resource-constrained settings. This is only possible through strategic leadership that recognizes the importance of these biological materials to the respective countries and communities from which they are collected. And for organizations requesting for these materials to recognize the efforts and cost of the collection, storage, and maintenance and follow the national and internationally recognized guidelines, rules and regulations regarding the sharing of the same.

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