Chapter 1

Field Isolation and Cultivation of Trypanosomatids from Insects

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Abstract

Monoxenous (one host) trypanosomatids from insects and other invertebrates can be introduced into axenic culture relatively easily and efficiently, allowing for their transfer from the field into the laboratory. Here we describe simple methods and alternative cultivation protocols, the wider application of which will allow substantial expansion of trypanosomatids available for research.

Key words Trypanosomatids, *Trypanosoma*, Cultivation, Field, Isolation, Axenization, Insects

1 Introduction

Trypanosomatid flagellates belong to the class Kinetoplastea Honigberg, 1963 emend. Vickerman, 1976, order Trypanosomatida Kent, 1880, which is best known for its human parasites responsible for African sleeping sickness (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*), and diverse leishmaniases (*Leishmania* spp.). Consequently, hundreds of strains belonging to the species of the dixenous genera *Leishmania* and *Trypanosoma* (and to lesser extent *Phytomonas* spp., which are plant pathogens) have so far been isolated from their vertebrate and invertebrate (vector) hosts, introduced into culture and many subjected to extensive research into their morphology, biochemistry, molecular biology, and parasite–host interactions. Indeed, in the databases there are now numerous assembled and annotated genomes for members of these two genera. Consequently, trypanosomes and leishmanias are among the best studied protists [1]. Until recently, we knew very little about parasitic flagellates that constitute their sister groups [2, 3]. However, it turns out that the human-infecting flagellates evolved from species that parasitize exclusively insects [4]. The available data strong point to the fact that the dixenous life cycle (alteration between a vertebrate host and an invertebrate

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vector, almost invariably an insect) of trypanosomes and leishmanias developed from the monoxenous life cycle (a single insect host) retained by the genera *Leptomonas*, *Crithidia*, *Blastocrithidia*, and others [5, 6]. Hence, the study of these neglected parasites of insects (mostly true bugs [Heteroptera] and flies [Diptera]) is more important for our understanding of the evolution of parasitism in vertebrate hosts than appreciated until recently.

Until about a decade ago, globally there were only a dozen or so species and/or strains of insect trypanosomatids available in culture [7, 8]. This has changed after increasing interest in this group of parasites and a protocol was developed that allows relatively straightforward transfer of these flagellates from the field into the culture. As a result of field trips to many tropical, subtropical, and moderate climate countries on all continents [6, 9–12], trypanosomatids were isolated from dissected insect specimens and subjected to limited DNA sequencing. For the purposes of phylogenetic analyses, usually several highly conserved genes have been sequenced. At present, hundreds of species/strains are available in cryobanks around the world; all of them stored potentially indefinitely in liquid nitrogen. With the advent of cheap and large-scale DNA sequencing, this wealth of strains will be useful for insight into the evolution and diversity of trypanosomatid protists.

2 Materials

1. Insect/arthropod traps like funnels, pitfall traps, bottle traps, various modifications of malaise traps, and UV light traps. Different nets, such as aerial, sweep, and aquatic nets and hand (or electrical) aspirators.
2. Various catching traps and instruments must be supplemented by other collecting tools for manipulation with obtained invertebrates. For example, soft forceps, fine-tipped forceps, and aspirators are routinely used; white pan (or white paper) help with invertebrate separation.
3. The captured insects/invertebrates should be kept alive individually in plastic/glass vessels/containers until dissection. For example, 1.5 or 2.0 mL plastic microtubes with perforated lids can be used for smaller specimens; large insect specimens should be kept in 5–10 mL vials.
4. Before dissection, specimens must be anaesthetized/killed in vessels with 70% ethanol and transformed into sterile physiological solution (e.g., saline solution for IV used in hospital). Before their sterilization in ethanol, ethyl acetate (ethyl ethanoate, EtOAc) can be used to anesthetize larger insect specimens or their groups, especially those which survive longer in ethanol.

5. Plate (e.g., glass slides) and tools for dissection (microforceps straight and/or curved, probes blunt and/or sharp, iris scissors, scalpel, needles—for preparation of these tools *see* Subheading 3.3).
6. Optional: Stereomicroscope (dissecting microscope) or some type of watch repair magnifying glasses. However, the basic dissection can be done with just naked eyes.
7. Burner (either Bunsen when gas is available or transportable burner running on 96% ethanol) for dissection tools and coverslip sterilization.
8. Blotting paper (absorbent toilet paper or paper tissue is sufficient).
9. Glass slides (presterilized) and coverslips (optimal size is 20 × 20 mm) for light microscopy.
10. Dissection of insects and any subsequent manipulation with the gut and other organs is done in sterile Insect Ringer's solutions or 0.6–0.86% NaCl solution.
11. Light microscope with ocular lenses of at least 10× magnification and objective lenses of at least 20× magnification. However, an objective of 40× magnification is advantageous, as well as phase contrast. It is also helpful to have a LED diode lighting with the accumulate battery to be independent on the electric current. If necessary, a head torch can be used as a light source.
12. Sterile insulin syringes (up to 1 mL) with permanently attached needle (sterile plastic tips for pipettes).
13. Cultivation is typically just one of several ways how to process the obtained material. Part of the dissected material is regularly used for microscopic slide smears (slides should be air-dried and fixed with methanol). For subsequent DNA extraction, homogenized dissected tissues are stored in plastic 1.5 mL microtubes with ethanol or (preferably) in 1% SDS + 0.1 M EDTA solution. Various standard protocols for DNA extraction are suitable, although "mini" kits are ideal due to the small amount of analyzed material. Isolated DNA is useful not only for parasite (primarily trypanosomatid) detection, but also for any other purposes (host barcoding, microbiome screening, etc.).
14. Primo-culture cultivation can be performed in different types of vials and media (*see* Subheading 3.7). Glass vials with rubber lids supplemented with biphasic media, which consists of the blood agar base and the liquid overlay. This approach provides more feasible conditions for finicky parasite species and is also suitable for isolation of trypanosomes or leishmanias from their vertebrate hosts. On the other hand, monoxenous trypanosomatids are very often able to grow even in much simpler and

nutritionally less rich monophasic media. Various cultivation media can be prepared using of several components (for more details *see* Subheading 3.7): Brain Heart Infusion (BHI), RPMI 1640 (with HEPES), complete Schneider's (Drosophila; Insect) Medium; M 199 Medium; heat-inactivated Fetal Bovine Serum (FBS), rabbit or sheep defibrinated whole blood, agar, neopeptone (or similar), 0.6% NaCl solution and various antibiotics (penicillin, amikacin or gentamicin, streptomycin, chloramphenicol, 5-fluorocytosine).

15. If axenization is required, glass V- or U-shaped tubes are recommended.
16. In case of cloning, a Bürker chamber or cell counter and 96-well plates with F (flat)-bottom are required.
17. For cryopreservation of trypanosomatid cultures, dimethyl sulfoxide (DMSO) and Mr. Frosty™ container (or a similar device) are commonly required.
18. An important aspect of some studies is the preservation of dissected host organisms for subsequent morphological determination, although other methods, such as DNA barcoding, can also be used. It is important to keep not only the insect body but also the genitals that are very often dislodged from the body during dissection. The best way is to store dissected specimens separately in plastic microtubes (1.5 or 2.0 mL) with 70% ethanol and labeled by an ethanol-resistant marker (permanent fine-lined paint marker; e.g., Edding 780). However, simpler and space-saving dry storage methods can be used; a plastic container with sheets of blotting paper for storing dissected specimens will do. In this case, a limited risk of specimen mixing has to be considered.

3 Methods

All methods described below are designed so that they can be performed both inside and outside of the laboratory, in a hotel room, in a porch, or many other locations. In the case of field work, the folding camping table and chair(s) are an important part of the equipment. Electricity is advantageous. However, in the field, use of a microscope (and optional stereomicroscope) with rechargeable batteries means electricity is not a necessity.

3.1 Catching Techniques

We will not describe here all methods used for trapping insects and/or other invertebrates that can be investigated for the presence of trypanosomatids. There is no universal capturing method, and one has to take into consideration the target group of insects/invertebrates, as well as the conditions in the field. Still, some

basic methods and principles are mentioned below. Invertebrates are passively caught using funnels, pitfall traps, bottle traps, and various modifications of malaise traps, some of which could be baited with different attractants. Several different types of light traps (ultraviolet lights ["black lights"], mercury vapor, and various others) represent active forms of catching. Physically more demanding but logistically much easier is the usage of different nets: aerial nets are used to collect flying insects; sweep nets (or beat nets) are suitable for sweeping the vegetation; aquatic nets are used to sample in the aquatic environments; litter reducer is designed for extracting soil invertebrates from the forest floor; beat sheets are suitable for collecting insects from branches of trees and shrubs. In specific situations (e.g., thorny forest, shrubs, feces, rooms inside houses) specimens are collected manually or with a hand (or electrical) aspirator.

3.2 Keeping the Hosts

Insects and other invertebrates can be captured by methods such as those described above, with the most applicable being sweep netting, different light trap methods or individual picking. For entomologic purposes, collected insects could be placed into different killing jars with ethyl acetate, the anesthetic we use most frequently. Importantly, for parasitologic purposes, the captured insects/invertebrates are kept alive as long as possible in any suitable plastic/glass vessel or vial prior to dissection. Depending on their size, the captured insects or other invertebrates are placed (preferably individually to prevent contamination by coprophilia or predation) into 1.5–2.0 mL plastic microtubes, or larger plastic or glass containers with pierced lid to allow access of air. In addition to the insect's size, one also has to consider several other aspects. For example, some true bugs (e.g., sting bugs of the superfamily Pentatomoidea) have glands that produce a foul-smelling liquid, which is used defensively to deter potential predators. However, if bugs are kept in a small space, they can kill themselves. Where this is a possibility, the container volume should be at least ten times the volume of the insect.

Insects in microtubes/vials and kept in shade and at room temperature normally survive for 24 h. If long-term storage (up to 1 week) is required, the insect containers should be stored in a refrigerator (4–10 °C). However, cooling of the container may result in precipitation of small droplets of water on its walls, which may lead to insects sticking to the wet walls, followed by aspiration of water into their tracheal system and subsequent drowning on the insect(s). To prevent this situation, adding a piece of blotting paper into vials may improve long-term survival of insects.

3.3 Dissection and Slide Preparation

The selection of dissection tools depends on several aspects: (1) insect size; (2) sclerotization of the cuticle; (3) the used optics (stereomicroscope versus naked eye), and (4) the experience of the

person who carries out the dissection and her/his capacity to perform fine work leading to the precise localization of trypanosomatid infection within the insect's organ systems (e.g., infection of digestive tract, hemolymph, or salivary glands). The most universal and preferred way for the dissection of most insects (e.g., hemipterans and dipterans of standard size) is the use of a pair of fine tweezers (microdissecting forceps); the harder and the thinner the spikes are the better. However, tweezer spikes can be easily damaged by careless handling or falling to the ground. Thus, it is advisable to carry several backup tweezers and/or forceps. If the insect's body is too hard (large and/or highly sclerotized specimen) or leather-like (e.g., hippoboscid flies), it is advisable to use for initial opening a small scalpel, which can be substituted by the slanted end of a syringe; this generally pierces and cuts through the body well.

If the investigator is not interested in localizing the infected organ, or is limited by other factors such as time, precise dissection (*see* Subheading 3.3) can be replaced by the following approach. The last one-third of the abdomen can be crushed and teared in a drop of saline solution into small pieces by using a pair of disposable fine-point wooden or bamboo toothpicks. Alternatively, if one wants to perform precise dissection of small insects (few millimeters long), dissection under a stereomicroscope is highly recommended. It is advisable to use special thin needles, such as the entomological pins (stainless steel; 1, 0, 00, or 000) or the minuten pins (stainless steel; 0.15 or 0.20 mm; but not 0.10 mm since these are too thin) set into a wooden (not bamboo) skewer. This tool can be prepared as follows:

1. After one end of the skewer dip has been submerged for a few hours in water, drive a minute pin (by its blunt end) by using a small pliers or forceps into the soft (= wet) end of the skewer.
2. Once the skewer has dried, the bonding can be enhanced by dipping with a glue. The obtained dissecting needles are relatively tough and durable and can be used as such. It is also possible to bend the tip third of the minuten into an "L" shape or into a small hook. These three shapes can be combined during dissection with commercial dissecting needles and tweezers or forceps.

Regardless of the technique and dissecting tools used (except disposable toothpicks), it is necessary to sterilize the tools after each dissection; ideally, clean them mechanically by wiping them into tissue paper to remove any stuck pieces of the dissected insects, shortly dip the tools into $\geq$70% ethanol and sterilize them over a flame from a small portable burner.

When everything is prepared for the dissection, the insect is removed from the container and dropped for a few seconds (or until it stops moving) into a small glass container (preferably

transparent) filled with 70% ethanol, where it will quickly drown. This step will kill the insect and sterilize its surface. However, if the specimen remains in ethanol for an extended period of time (more than few minutes), ethanol can penetrate the body and kill intestinal and other endoparasites including trypanosomatids. Next, the dead specimen is taken out, fast-dried on a piece of filter or tissue paper, and dropped into another small glass container with sterile physiological solution. Taking into account the location, date and other "sorting" information, it is possible to perform this operation (ethanol—drying—sterile solution) with several specimens at the same time. The killed insects can stay in the container with physiological solution for several hours; it is even possible to keep them there overnight (e.g., when it is not possible to finish the work). In this case, it is preferable to leave a container with nondissected insects in the refrigerator. The sterile solution and containers can be transported to the laboratory or prepared in situ from a preweighed quantity of salt mixed with water (even drinking water is acceptable). In the field, sterilization can be done, for example, by use of a kettle.

Except for very large insects, a sterile microscopic slide (presterilized from the lab or sterilized over a flame of a small portable burner) is used as a dissecting pad. Before dissection, two or three drops of sterile physiological solution (from a syringe, plastic dropper or, optimally, from a plastic dropping ampulla with sterile NaCl solution) are applied on the slide. Next, the insect kept in a container with sterile physiological solution is transferred onto a slide. It is essential to minimalize the volume of transferred solution from the storage container along with the insect—especially sting bugs release various chemicals (terpenes, etc.) that change the water tension and repel droplets of solution in which the dissection has to take place.

Next, the insect is placed in the supine position. Sometimes it is recommended to remove legs and wings before the dissection. However, since the insects need to be preserved for later morphological determination and also because of the considerable workload of this step, we recommend leaving the legs and wings. A slightly different situation concerns the head. In some insect groups with a bigger head that is loosely connected to the body (e.g., flies and cockroaches), separation of the head is necessary before removal of the intestine. On the other hand, in insects with a relatively small head firmly attached to the body (e.g., true bugs, some beetles, and fleas); separation of the head is unnecessary and, in addition, can be very laborious. After decapitation (optional), the last two or three segments of the abdomen must be removed and gently separated from the rest of the body. Depending on the dissected insect, the digestive tract is more-or-less firmly attached to this abdominal segment and can be pulled out from the body.

Several different situations may occur during the dissection. The ideal situation can be described as follows: together with the separated apical abdominal segments, pull out as one piece the entire digestive system—hindgut (HG), Malpighian tubes (MT), and whole midgut (MG) including cardia (stomodeal valve; stomodeum–mesenteron connection) and diverticules (crop). This is the fastest way and it is highly likely that all parts of the digestive system have been acquired. Moreover, if the intestine remains intact, it is easy to determine its anatomy and distinguish its different parts (HG, MG, crop). Unfortunately, the digestive tract often breaks, most frequently at the hindgut–midgut junction. In such cases, the tweezers or the dissecting needle must be pushed into the abdomen in order to pull out the rest of the digestive system. Sometimes, however, the digestive tract has to be pulled out in several parts. We provide two tips how to improve the dissection and increase the chances of pulling out the entire digestive tract: (1) in case of females, when last abdominal segments are further apart from the body (but still connected with the intestine), remove eggs or developing ovaries from the body cavity because these parts of the insect's tissue block the abdominal opening and make it difficult for the intestinal tract to be pulled out; (2) when pulling out the intestine, it sometimes helps to "massage" the body from the outside in the head-to-abdomen direction.

After removal of the intestine from the body cavity, proceed by separating all other tissues, especially the reproductive organs. Indeed, in females the sclerotized eggs complicate the covering and smashing of the intestine with a coverslip, while the ovaries shadow the microscopic field. In the case of males (or spermatheca in females), the movement of sperm released into the observed sample complicates the detection and observation of the parasitic flagellates. The last two abdominal segments, still attached to the intestine, must be removed as well (exept very small specimens, e.g. flies); otherwise they prevent proper covering and smashing by the coverslip and shadow some parts of the intestine, especially the rectal ampule area. In addition, the male genital organs, such as the aedeagus, are often essential for morphological determination. Removal of these abdominal segments, however, must be done very carefully in order to retain as much of the hindgut as possible, including the rectal ampule.

After removal and careful cleaning, it is recommended to transfer (by tweezer, needle, etc.) the whole intestine to the second (= clean) drop of saline solution on another slide. If the intestine is pulled out in small pieces, it is better to keep it in the "dissecting drop" of the saline solution, from where one shall remove the rest of the body and all the contaminants. The intestine shall be inspected in one piece, as long as it is kept in a stretched-out formation. For bigger specimens, it is better to separate the hindgut + rectal ampule (HG + RA) and midgut + Malpighian tubes

(MG + MT), push these two parts aside and cover them gently by separate coverslips, yet still on the same slide. The slide is large enough to fit three drops: a "dissecting" drop (with insect remnants), a coverslip with HG + RA and a second slip with MG + MT. Afterward, the slide is ready for inspection under the microscope. If the slide is not immediately examined, it should be stored in a humid chamber (food chamber, kitchen dishes, braced plastic bag, etc.) to prevent it from drying out.

For accuracy and completeness, we shall add that the digestive system is not the only tissue that can be infected and henceforth checked for trypanosomatid parasites. For dixenous species (especially trypanosomes and *Phytomonas* spp.), the examination of salivary glands may provide important information about possible vector capacity of the inspected insects. However, dissection of the salivary gland is much more difficult than dissection of the intestine and differs among insect groups. Dissection of the salivary glands is practically impossible with the naked eye and a stereomicroscope, preferably supplemented with transmitted light, is therefore indispensable.

3.4 Light Microscopy

The slide is then inspected under a microscope, preferably using magnification around 100–200× (10× oculars combined with a 10× or 20× objective), which allows for monitoring any moving objects in a relatively large area.

1. First, check for a few minutes the objects under the coverslip without trying to crush the intestine. This will allow precise determination in which part and where inside the intestine the parasites are localized.
2. After the primary examination of the intact intestine, its contents are squeezed by a gentle pressure (e.g., by using tweezers) onto the coverslip, and the homogenate observed again for a few minutes.
3. When the twisting behavior of (usually) slender cells is noticed, switch to 40× objective and eventually use the phase contrast for better observation of the flagellum and other morphological features. This allows for unequivocal distinction of fast-moving sperm cells or elongated bacteria from the trypanosomatids. Once parasitic flagellates have been found in any part of the insect (if this happened before squeezing by the coverslip, it is important to smash the intestine content thoroughly), using tweezers to remove gently the coverslip, add a drop of saline solution or growth medium to the homogenized tissue pieces and carefully mix the content by repeated ingestion and extrusion through the insulin syringe. This will increase the volume of fluid available from the dissected specimen. A sterile insulin syringe (up to 1 mL) with permanently attached needle is

particularly suitable. Using the detachable needle is more laborious and in the case of a very small volume, more material remains in the syringe–needle connection. For some applications, insulin syringes can be replaced by a sterile plastic tip (for pipettes), although the former are more practical. By using a cap, the dissected content can be kept (semi)sterile inside the barrel part of the syringe and tender movement of the plunger will allow homogenizing the content and splitting it up for several application (*see* Subheading 3.5).

3.5 Division of the Sample

It is important to preserve the trypanosomatid morphotypes as they occur within the host. The cultures obtained from the infected samples frequently do not reflect the original situation, either because the morphology changes under specific cultivation conditions, or because only one species prevails from what was originally a mixed infection. Hence, upon detection of a trypanosomatid infection, the infected and homogenized tissue material from the slide is taken up into an insulin syringe and divided into three parts, which are used as follows (for more details also *see* Subheadings 3.6, 3.7 and 3.11): (1) about 1/3 to 1/2 is injected into a vial for primo-culture (start with this step to decrease the chance of contamination); (2) one or two drops are used for a slide smear; (3) the remaining sample is stored for later DNA extraction.

3.6 Preparation of Slides

For morphological inspection, place a small aliquot of the diluted sample on a microscopic slide and using a tilted coverslip (or another slide), make a thin smear (like a blood smear) and allow it to dry. The usage of growth medium (especially if it contains fetal bovine serum) improves the adherence of the parasites to the slide. In a humid environment, the slide may not get dry fast enough. In such case, put the slide for example on the warm part of the microscope and let it dry there. Even dry slides can attract insects (mostly flies) to graze on them and destroy a significant part of the smear. In order to prevent this, describe the slide with an ethanol-resistant marker, place the dry smear into a horizontal position and cover it with a few milliliters of methanol or submerge the slides in vertical position into some container with methanol (can be used repeatedly) for about 5 min. Let the slides dry and afterward place them into a microscopic slide container (with silica gel if there is high humidity) for transportation and further use in the laboratory. There, put the dry slide horizontally onto a rack or vertically into a staining jar and cover it with Giemsa stain (diluted 1:10–20 with water) for 15–30 min, and remove the stain by rinsing the slide under running water. Giemsa is still the golden standard, but several other staining methods (e.g., diff-quick) could be used. Air dried smears are ready for inspection under the microscope. It is practical to later carefully inspect only slides, for which either or both cell culture or DNA sequences are available.

Note: if methanol is not available in the field, dried slides may be kept without fixing for several weeks; however, it is necessary to keep the slides in a dry place to prevent their molding or destruction by any other (micro)organisms.

3.7 Preparation of Vials and Media for Primo-Cultures

In the era of whole genome/transcriptome/proteome sequencing, one should attempt to bring as many trypanosomatid parasites as possible into culture. As they can usually be easily perpetuated in culture as well as cryopreserved, once established the culture can be used for further research and easily shared among laboratories. It will also make possible ultrastructural examination, infection experiments, and other studies.

There are several ways how to transfer parasites inhabiting the gut and/or other organs of their invertebrate hosts into the culture. Some monoxenous trypanosomatids (e.g., species of the genus *Leptomonas*) are very amenable for cultivation and even nutrient-poor and simple media are fully sufficient for their maintenance. Since the cultivation of trypanosomatids from the dissected invertebrate hosts is often plagued by contaminations with various bacteria and/or fungi, the selection of nutritionally poor media has a great advantage in terms of suppressing the growth of these contaminants. On the other hand, some trypanosomatids, mainly belonging to the genus *Blastocrithidia*, grow very slowly and richer and more complex monophasic or biphasic media are required. Finally, it must be mentioned that in some cases (e.g., some species of the "jaculum" group), cultivation is very difficult if at all possible, and thus, so far, all attempts have failed. We have considered the possibility of cultivating these elusive trypanosomatids in insect tissue cultures, but keeping vials with these rather sensitive cultures in proper conditions during the field work is logistically very difficult and also any type of bacterial and fungal contamination can easily overgrow the culture. Taking into account all the above-described limitations and problems, it seems most advantageous to choose a nutrient-rich monophasic medium. Still, several other options will be described below.

In the case of a monophasic medium, sterile plastic 1.5–2.0 mL microtubes are prefilled (max 1.0 mL of medium for a 1.5 mL tube and 1.5 mL of medium for a 2.0 mL tube) in the laboratory and shall be kept at 4 °C before use, preferably within 1 or 2 months. However, if this is not possible, the vials with media keep ready-to-use for a few weeks even in room temperature. The nutrient-rich monophasic growth medium of Brain Heart Infusion (BHI) works most frequently and therefore represents the first choice. Although the liquid form of BHI is also available, powdered medium is the most common form of this commodity. Dissolve 37 g per liter of distilled water, heat it, and stir until completely solubilized. Dispense it into an appropriate container, loosen the cap, and autoclave for 15 min at 121 °C. After cooling, supplement the medium with

10 mg/mL hemin and 10% of heat-inactivated (30 min at 56 °C) fetal bovine serum (FBS). The supplementation with 0–5% FBS represents the less nutrient-rich variation. All media must be supplemented with an antibiotic cocktail (see below). A more nutrient-rich medium shall be prepared by using several commercially available growing cultivation media (Gibco, Sigma-Aldrich, etc.). One option is a mix of RPMI 1640 (with HEPES), Complete Schneider's (Drosophila; Insect) Medium and M 199 Medium (1:1:1) supplemented with 10% FBS and antibiotics (see below).

The preparation of biphasic blood agar media is more complicated. For the blood agar base, 2% agar, 2% neopeptone (or similar), and 0.6% NaCl water solution is prepared and autoclaved (15 min at 121 °C). Being shaken gently, the solution is left to cool to approximately 60 °C. Then, rabbit or ovine (but also other blood sources are usable) defibrinated blood is added to a final 20% concentration, thoroughly mixed and the glass vials are filled as quickly as possible to prevent the solidification of agar in the pipette and/or in the bottle (the bottle with liquid blood agar shall be kept in a thermally insulated tank with hot water). Different types of glass vials can be used. A good choice in terms of storage and ease of handling are narrow 4–5 mL glass vials with a rubber cap (Fig. 1)—different types of capping can be used: the most common is top jaw (requiring sealing jaw capping machine); however, a screw or bayonet cap are also suitable. Open sterile vials are filled with 0.5 mL, agar is poured onto the bottom end and the vial is immediately closed by the sterile rubber lid and placed into a horizontal position where agar is allowed to spread over the side wall to maximize surface coverage (optimally, the agar shall cover one wall from the bottom end of the vial to the cap). To avoid solidification of the agar, it is necessary to work fast and at the same time stay sterile; a heated flow box is a good solution.

Fig. 1 A biphasic blood agar medium (0.5 mL of blood agar solidified in horizontal position) and liquid phase overlay stored in narrow 5 mL sterile glass vials closed by a rubber cap. A sterile insulin syringe with permanently attached needle is used for injection of the sample by piercing a rubber cover of the glass vial containing the overlay and the solid phase

Agar base represents the solid phase, and various liquid phases (overlays) can be used. The less nutrient-rich overlay consists of 2% neopeptone and 0.6% NaCl water solution (autoclaved). Alternatively, the most complex and nutrient rich medium contains a mix of complete RPMI 1640, Schneider's and M199 (1:1:1) supplemented with 2% human urine (sterile; filtered using 0.2 μm pore filters) and 10–20% heat-inactivated FBS. Do not mix overlay with blood agar before use. Similar to blood agar, the overlay is stored in 4–5 mL vials with a rubber cap (Fig. 1). The vials with blood agar shall be stored at 4 °C until use, preferably within 1 or 2 months.

Crucial and really fundamental for the success of primocultures is the use of antibiotics, regardless of the selected growth media (see above; in case of autoclaving, antibiotics must be added after autoclaving, when the solution already cooled down). In general, potential contaminants (bacteria and fungi) grow faster in nutritionally rich media. In any case, antibiotics need to be used even in less rich growth media. Unlike the cultivation from the more-or-less sterile blood or tissues from vertebrates (for *Trypanosoma* and *Leishmania* species) or tissues from plants (for *Phytomonas* species), the monoxenous trypanosomatids of insects or other invertebrates are mostly derived from the intestinal tract and are therefore contaminated with a large number of microorganisms. It is advisable to use a mix of different antibiotics in a fairly high concentration (higher than is commonly used under laboratory conditions). The following combination is generally suitable: 10,000 IU penicillin, 100 μg/mL amikacin or gentamicin, 100 μg/mL streptomycin, 50 μg/mL chloramphenicol, 1500 μg/mL 5-fluorocytosine.

The use of antibiotics, however, brings one possible limitation. Different obligatory endosymbiotic bacteria have been found in the cytoplasm of monoxenous trypanosomatids belonging to the genera *Strigomonas*, *Angomonas*, and *Kentomonas* [13, 14], as well as in the genus *Novymonas* [15]. Although the abovementioned antibiotics do not affect the endosymbiotic bacteria in these monoxenous trypanosomatids, it cannot be ruled out that in some cases the use of antibiotics may lead to the elimination of endosymbionts and thus either failed cultivation or overgrowth of symbiont-free trypanosomatids, which do not correspond to the natural situation.

In the field, inject about 1/3 to 1/2 of the sample (diluted with a drop of saline solution or growth medium from a sterile insulin syringe) into a vial. This shall be done by opening the microtube with the medium or piercing a rubber cover of the glass vial containing the medium and solid phase. When more types of media are available, using the same syringe to inject roughly equal amounts into each type of medium. At the same time, leave enough sample volume for a slide smear and DNA extraction. After a gentle mix, leave the glass vial with blood agar in horizontal position, while the

microtube may be kept in vertical position. All types of media with injected parasites must be kept and transported in shade at room or lower temperature. Especially when in the field, it is really crucial to ensure that the cultivation vials/tubes will never be exposed to high temperature (e.g., when a bag is left in the sunshine, is close to an engine or driveshaft cover in a bus/boat). During prolonged field expeditions, it is advisable to keep the cultivation vials/tubes at lower temperature (10 °C is optimal, but 4 °C is also possible) or passage them each 2 weeks. If the vials/tubes are stored for an extended period of time, it is recommended to shake and mix them occasionally, especially when the microtubes are kept in boxes in vertical position. The surface of the growth media may become covered by fungi, which prevents oxygen from entering the lower part of the liquid column and thus aerobic organisms, trypanosomatids can die from anoxia.

3.8 Axenization

Upon transportation into the laboratory, the next steps depend on whether the cultures have remained sterile or whether they have been contaminated by bacteria and/or yeast (see below). In any case, following the return from the field, attend to the cultivation vials first. Check them for the presence of trypanosomatids as well as contaminants under a microscope with magnification 200–400× (phase contrast is advisable) up to one (two) month after the dissection, in 1-week intervals. In case of glass vials with a rubber lid, which were kept in horizontal position, mix the content thoroughly and using an insulin syringe, transfer a small drop on the slide for inspection. In case of the microtubes, which were kept in vertical position, for the same purpose take carefully a small drop from the surface of the medium.

Although the media contain a mix of antibiotics, at least in some vials a contamination with bacteria and/or fungi will usually occur. There are several ways how to deal with the contaminants and axenize (= separate particular organism from all others) the trypanosomatids growing in the primo-culture. If a given trypanosomatid grows well even in a nutrient-poor medium, fast passages may be sufficient to remove the contaminating yeast. However, this approach is applicable only for yeast contaminants, as it does not work in the more frequent case of bacterial contamination. One possibility is to eliminate bacteria by the addition of another mix of antibiotics.

However, when bacteria become quickly resistant to the newly added antibiotics, which is frequently the case, the next method of choice is the migration technique through a glass V- or U-shaped tube (Fig. 2). A sterile tube is filled with a growth medium about 3 cm above its bottom (V or U bent), and about 500 μL of the contaminated culture is added into one arm. Next, the openings are plugged with cotton wool stoppers (or other type of sterile cover), and the tube is fixed in an upright position. Within a few hours (no later than overnight) a few drops of the medium from the

Fig. 2 A U-shaped sterile glass tube filled with a cultivation medium is used for axenization of cell culture contaminated with bacteria or yeast, which is based on migration technique (it utilizes the higher motility of trypanosomes as compared to that of contaminating microorganisms)

surface in the opposite (i.e., noninoculated) arm shall be checked for the presence of trypanosomatids. The motile protists shall actively migrate through the narrow base of the V- or U-shaped tube, while the nonmotile (or by Brownian movement only slowly progressing) contaminants remain behind. It has to be kept in mind that if trypanosomatids grow poorly or do not replicate at all, or if the contaminating microorganisms are also mobile, this method is ineffective. In some very specific cases other axenization techniques can be applied, such as the repetitive purification of trypanosomatids via a Ficoll gradient, cultivation on solid agar media, culture cloning (*see* Subheading 3.9) or different combinations of the abovementioned methods.

3.9 Culture Cloning

A single clone can be obtained from an axenic culture by in vitro cloning. Prior to the initiation of in vitro cloning, several subpassages of the culture are made. This is done by transferring a few drops of the trypanosomatid suspension to a new culture flask (in a ratio 1:10 maximum) at 3- to 10-day intervals (depending on the growth rate). Next, the cell density of an exponentially growing culture has to be established, at least approximately, using a Bürker chamber or cell counter, and the culture is diluted with a fresh preconditioned medium to the density of about 2 cells/mL. It is then pipetted into one to three 96-well plates with F (flat)-bottom (150 μL per well). It is advisable to add into the 96-well plates a preconditioned medium, which is prepared as follows. Cultivation medium from a culture of exponentially growing trypanosomatids

is filtered through 0.22 μm filter; the conditioned medium contains undefined secreted growth factors that usually significantly improve the growth rate of the single cloned cells. Using an inverted microscope, in 1-week intervals the plates are screened for the presence of cells. Usually a clonal expansion in at least a few wells occurs within 30 days.

3.10 Cryopreservation

Cryopreservation of trypanosomatids is relatively easy and straightforward. An exponentially (preferably but not necessarily) growing culture is mixed with dimethyl sulfoxide (DMSO) at the final concentration of 5–10%. Due to the inherent toxicity of this cryoprotectant, after defreezing the mix of the cryopreserved culture is diluted with fresh medium at a ratio of at least 1:10. Alternatively, instead of DMSO, glycerol at the final concentration of 30% can also be used. In this case, it is crucial to leave the culture mixed with glycerol for about 15 min before the freezing procedure has been started. This is because glycerol is known to enter the cells significantly less rapidly than DMSO. After mixing with DMSO, the culture must be immediately transferred into the cryopreservation tubes (at least 5 tubes per culture are suggested) and allow gradual decrease of the temperature at a rate of about 1 °C per minute. The simplest way is to use the cheap Mr. Frosty™ container, by placing it along with the tubes into −80 °C deep freezer and leave it there overnight. Next day, the tubes are transferred into a liquid nitrogen tank. When liquid nitrogen is not readily available, trypanosomatids are known to survive cryopreserved for several months even in the deep freezer at about −80 °C. It is advisable to verify the success of the cryopreservation by defreezing one tube next day after the cryopreservation.

3.11 DNA Analysis

Generally, the trypanosomatid flagellates have very few conspicuous morphological characteristics observable by light microscopy that would allow their determination. This makes sequence analysis indispensable [3]. We provide protocols for the PCR amplification of 18S ribosomal RNA (rRNA) [3, 4, 10, 11, 13] and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) genes [16] that are available from hundreds of trypanosomatid species and their sequence will thus allow unambiguous determination to the generic and species level. Another possibility, suitable for taxonomic identification within a genus, is to use the spliced leader (SL) RNA gene sequences [9, 17, 18].

In the field, approximately 1/3 to 1/2 of the dissected intestinal material containing trypanosomatid parasites is preserved for later DNA extraction. Using an insulin syringe, this material is put into a 1.5 mL safe-lock plastic microtube containing 1% SDS and 0.1 M EDTA for tissue lysis and DNA preservation. The volume of SDS + EDTA should be 5–10 times of the volume of the added dissected material and the tube is gently mixed. A labeled tube (by permanent fine-lined paint marker) is ready for transportation.

During transportation, it is advisable to add some tissue paper into the box with tubes, which will absorb the effluent occasionally released from a broken or improperly closed tube. Tubes should be kept in a refrigerator (4 °C) or freezer (−20 °C) whenever possible, and at −20 °C upon delivery to the lab. Still timing is not critical, as in most cases the samples will survive at room temperatures for weeks.

Note: Instead of SDS + EDTA, RNAlater solution can also be used. If none of these solutions is available, sterile physiological solution or PBS can be used instead, but in that case the samples have to be kept frozen for the whole time. Another option is to use ethanol (final concentration 50–100%), but one has to keep in mind that DNA extraction and PCR from ethanol-stored samples may be less efficient.

In the laboratory, briefly vortex the tubes and from the total amount of preserved material, use about 2/3 (to have a backup (1/3) in case the downstream methods failed). Whenever cultures have been successfully established, use about 10^7 cells or less for DNA extraction. Any standard protocols for DNA extraction is suitable; in the case of very small amount of material, various "mini" or "micro" kits shall preferably be used.

To establish taxonomic appurtenance and phylogenetic position, the gene of choice is 18S rRNA with the following primers (forward, AACCTGGTTGATYCTGCCAGTAG and reverse, TGATCCWKCTGCAGGTTCACCTAC) using this PCR program:

94 °C for 3 min.

35 cycles: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s.

72 °C for 10 min.

This protocol is perfectly suitable for DNA samples obtained from cultured flagellates. However, in cases of the environmental sample the application of the nested PCR protocols for 18S rRNA amplification is recommended.

1st PCR: Forward (S763), CATATGCTTGTTTCAAGGAC and reverse (S762), GACTTTTGCTTCCTCTADTG.

94 °C for 3 min.

40 cycles: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s.

72 °C for 10 min.

2nd (nested) PCR: Forward (TRnSSU-F2), GARTCTGCG-CATGGCTCATTACATCAGA and reverse primers (TRnSSU-R2), CRCAGTTTGATGAGCTGCGCCT.

94 °C for 3 min.

35 cycles: 94 °C for 30 s, 64 °C for 30 s, 72 °C for 90 s.

72 °C for 10 min.

The second gene of choice is gGAPDH (or other highly conserved genes), amplifiable using one of the below protocols.

gGAPDH protocol I: Forward (M200), ATGGCTCCVVTCAARGTWGGMAT and reverse primers (M201), TAKCCCCACTCRTTRTCRTACCA.

94 °C for 3 min.

35 cycles: 94 °C for 30 s, 55 °C for 45 s, 72 °C for 90 s.

72 °C for 10 min.

gGAPDH protocol II: Forward (GAPDH-F), ATGGCTCCGMTCAAGGTTGGC and reverse primer (GAPDH-R), TTACATCTTCGAGCTCGCGSSGTC.

94 °C for 3 min.

35 cycles: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s.

72 °C for 10 min.

This protocol is perfectly suitable for DNA samples obtained from cultured flagellates; however, it works (with some limitation) also for the environmental samples.

4 Notes

In the last two decades, dozens of new species of monoxenous trypanosomatids have been described and formally named. However, at the same time, a large number of most likely new species were discovered but remain undescribed. We and our collaborators have decided to formally describe (and named) only those trypanosomatids for which either an axenic (cryopreserved) culture is available, or those which frequently parasitize (and/or with high prevalence) widely distributed insect hosts [19], or finally those flagellates that infect hosts kept under laboratory condition.

All other species identified by DNA sequencing and/or other methods remain formally taxonomically undescribed (= unnamed). For that reason, typing units (TUs) are used as proxies of monoxenous trypanosomatid species [3], which are similar to the molecular operational taxonomic units (MOTUs).

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