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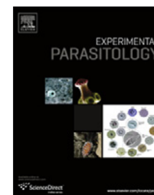

Trypanosoma brucei gambiense: HMI-9 medium containing methylcellulose and human serum supports the continuous axenic *in vitro* propagation of the bloodstream form

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ABSTRACT

Trypanosoma brucei (*T.b.*) *gambiense* causes the chronic form of human African trypanosomiasis or sleeping sickness. One of the major problems with studying *T.b. gambiense* is the difficulty to isolate it from its original host and the difficult adaptation to *in vivo* and *in vitro* mass propagation. The objective of this study was to evaluate if an established method for axenic culture of pleomorphic bloodstream form *T.b. brucei* strains, based on methylcellulose containing HMI-9 medium, also facilitated the continuous *in vitro* propagation of other bloodstream form *Trypanozoon* strains, in particular of *T.b. gambiense*. Bloodstream form trypanosomes from one *T.b. brucei*, two *T.b. rhodesiense*, one *T. evansi* and seven *T.b. gambiense* strains were isolated from mouse blood and each was concurrently cultivated in liquid and methylcellulose-containing HMI-9 based medium, either with or without additional human serum supplementation, for over 10 consecutive sub passages. Although HMI-9 based medium supplemented with 1.1% (w/v) methylcellulose supported the continuous cultivation of all non-*gambiense* strains better than liquid media could, the *in vitro* cultivation of all *gambiense* strains was only achieved in HMI-9 based medium containing 1.1% (w/v) methylcellulose, 15% (v/v) fetal calf serum and 5% (v/v) heat-inactivated human serum.

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1. Introduction

Trypanosoma brucei gambiense causes the chronic form of human African trypanosomiasis or sleeping sickness, one of the most neglected diseases affecting rural populations in sub-Saharan Africa (Simarro et al., 2008). Although very closely related to *T.b. brucei* and *T.b. rhodesiense*, this parasite subspecies has its peculiarities of which the underlying mechanisms remain unknown, for example its restricted host range, its consistent normal human serum resistance and its differential virulence in human and laboratory rodents (Gibson, 1986). One of the major problems with studying *T.b. gambiense* is the difficulty to isolate it from its original host and the difficult adaptation to *in vivo* and *in vitro* mass propagation.

Direct isolation from the tsetse fly and the mammalian host has been achieved using the Kit for *In Vitro* Isolation (KIVI) that supports the growth of procyclic trypomastigotes (Aerts et al., 1992). However, KIVI is selective for certain subpopulations of *T.b. gambiense* (Jamonneau et al., 2003). To obtain the bloodstream form trypomastigotes from procyclic populations, the trypanosomes have to be passed through tsetse flies and trypanosome susceptible rodents, a particularly cumbersome manipulation with low success rate (Ravel et al., 2006). Thus, to produce sufficient quantities of bloodstream forms, it is preferred to isolate and propagate *T.b. gambiense* bloodstream forms immediately, either *in vivo* through rodent inoculation or via *in vitro* culture.

On isolation of bloodstream form *T.b. gambiense* in rodents, some recent improvements have been made. Büscher and co-workers reported higher isolation success rates in the thicket rat *Grammomys surdaster* than in *Mastomys natalensis* or immunosuppressed laboratory mice or rats (Büscher et al., 2005). Yet, in most instances

Abbreviations: HMI-9, Hirumi's modified Iscove's medium 9; HF, HMI-9 without Serum Plus but with 15% (v/v) of fetal calf serum; HH, HF with an additional 5% (v/v) heat-inactivated human serum; HFM, HF with 1.1% (w/v) methylcellulose; HHM, HH with 1.1% (w/v) methylcellulose.

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it is not feasible to bring these rodents close enough to place of diagnosis or treatment to allow direct inoculation of the patient's specimen. This problem has been solved with the introduction of a new cryomedium for *in situ* cryopreservation of blood from *gambiense* patients prior to inoculation of rodents in the laboratory (Maina et al., 2007).

For adaptation of *T. brucei* bloodstream form trypomastigotes to *in vitro* culture, diverse methods have been described, mostly making use of various combinations of media ingredients and animal sera and of feeder layer cells during the initial passages (Hirumi et al., 1977; Duszenko et al., 1985; Baltz et al., 1985). Hirumi and Hirumi succeeded in formulating an axenic medium for continuous cultivation of *T. brucei* (Hirumi's modified Iscove's medium, HMI-9) but adaptation to this medium invariably induces a period of massive cell death and hence a selection can occur before a continuous culture is established (Hirumi and Hirumi, 1989). In contrast with what is observed in liquid axenic cultures, primary cultures established using solid HMI-9-agarose plates do not suffer from massive cell death at the initiation stage (Vassella and Boshart, 1996).

Vassella and co-workers described that HMI-9 based medium containing low melting point agarose or methylcellulose, providing a high molecular weight matrix, allows several pleomorphic *T.b. brucei* strains to proliferate without aberrant cell division or growth arrest. Apart from culture and cryostabilisation, these media are also compatible with transfection of bloodstream form trypomastigotes (Vassella et al., 2001; McCulloch et al., 2004). So far, experiments on axenic *in vitro* culture in media containing methylcellulose have not yet been conducted with *T.b. gambiense*.

We investigated the suitability of HMI-9 based medium containing methylcellulose for the continuous axenic *in vitro* propagation of bloodstream form *T.b. gambiense* strains. In addition, we evaluated whether supplementation of this viscous culture medium with human serum improved the *in vitro* propagation of *T.b. gambiense* strains further.

2. Methods

2.1. Ethics statement

All experiments on animals were approved by the Institute of Tropical Medicine's Animal Ethics Committee under license PAR010-PB-MR-NEUROTRYP.

2.2. Culture media

Iscove's modified Dulbecco's medium powder and fetal calf serum (heat-inactivated EU origin) were purchased from Invitrogen. Methylcellulose (5140 mPa S) was purchased from Fluka. All other ingredients were from Sigma–Aldrich. A recipe to prepare a concentrated HMI stock solution, without serum, was adapted from McCulloch (McCulloch et al., 2004). This HMI stock solution was used to prepare four different culture media (Table 1). HMI – fetal calf serum (HF) is a close variant of HMI-9 but without Serum Plus and with a higher concentration (15% v/v) of fetal calf serum (FCS). HMI – fetal calf serum – methylcellulose (HFM) is HF containing a final concentration of 1.1% methylcellulose. HMI-human serum (HH) contains (15% v/v) FCS and 5% (v/v) heat-inactivated human serum. HMI – human serum – methylcellulose (HHM) is HH containing a final concentration of 1.1% (w/v) methylcellulose. The final concentration of methylcellulose in HFM and HHM medium was obtained by mixing the concentrated HMI stock solution with a 3% (w/v) methylcellulose solution. This concentrated methylcellulose solution was prepared in distilled water at 65 °C under continuous stirring followed by steam sterilisation for 20 min at 120 °C and rapid cooling in an ice bath under vigorous magnetic stirring (McCulloch et al., 2004).

Table 1
Recipe for 1 l of each of the HMI-9 variations[a].

Ingredient	HF	HH	HFM	HHM
2 × Iscove's modified Dulbecco's medium	365	365	365	365
100 mM hypoxanthine in 0.1 N NaOH	10	10	10	10
5 mM bathocuproine disulphonic acid	10	10	10	10
20 mM β-mercaptoethanol	10	10	10	10
16 mM thymidine	10	10	10	10
100 mM pyruvate	10	10	10	10
100 mM cysteine	10	10	10	10
Fetal calf serum	150	150	150	150
Human serum	0	50	0	50
H_2O	425	375	50	0
3% (w/v) methylcellulose	0	0	375	375

[a] All ingredients are given in milliliter per liter.

2.3. Trypanosomes

One *T.b. brucei*, two *T.b. rhodesiense*, one *T. evansi* and seven *T.b. gambiense* strains were used for the *in vitro* culture experiments. Original host, country and year of isolation and number of passages in rodents are given in Table 2. All *T.b. rhodesiense* and *T.b. gambiense* stocks originated from patients.

To obtain a population of bloodstream form trypomastigotes, female OF-1 mice (Charles River, Belgium), immune suppressed with 200 mg/kg cyclophosphamide (Endoxan, Baxter) 24–48 h before infection, were infected intraperitoneally with $1–10 \times 10^6$, parasites from a cryostabilate diluted 1:1 in phosphate buffered saline glucose pH 8.0 (PSG) (Lanham and Godfrey, 1970). The matching method was used to monitor parasitemia in tail-blood (Herbert and Lumsden, 1976). At first peak parasitemia, when the blood contained $1–10 \times 10^6$ ml^{-1} long slender bloodstream form trypomastigotes, the mouse was anaesthetised and its chest was disinfected with 70% ethanol. Blood was collected by cardiac puncture with a heparinised 1 ml syringe, transferred in a 15 ml tube near an open flame and centrifuged at 1000g for 15 min at 25 °C to concentrate the trypanosomes in the buffy coat. After centrifugation, the buffy coat was gently resuspended in the overlaying plasma, taking care not to disturb the red blood cell sediment. This parasite suspension in plasma was used to initiate *in vitro* cultures.

2.4. In vitro culture

The suspension was transferred to an equal volume of HF and the parasites were counted and diluted to a concentration of 1×10^5 cells ml^{-1}. Several primary cultures were initiated at 1×10^4 cells ml^{-1} by inoculating 50 μl of this suspension in 450 μl of medium in a 24-well plate. Cultures were incubated at 37 °C and 5% CO_2 and monitored every 24 h by phase contrast inverted microscopy or counting in disposable counting chambers (Uriglass, Menarini). To establish continuous cultures, primary cultures showing a density increase in the range of $1–10 \times 10^5$ cells ml^{-1} were sub passaged to densities of $1–10 \times 10^4$ cells ml^{-1} using at least 80–90% fresh medium. A culture was considered continuous when 10 consecutive sub passages could be made.

2.5. Cryostabilisation

Adapted cultures were scaled up tenfold in a 6-well plate format by inoculating 4.5 ml fresh medium with 500 μl of an exponentially growing culture. When the parasite density reached 1×10^6 cells ml^{-1}, 5 ml of the culture was diluted with 20 ml

Table 2
List of *Trypanozoon* strains used and their characteristics.

Taxon	Strain	Original host	Country and province or town	Year	Number of prior passages in rodent	Reference
T.b. brucei	AnTat 1.1^E	Bushbuck	Uganda, Busoga	1966	10	Le Ray et al. (1977)
T.b. rhodesiense	RUMPHI	Human	Malawi, Rumphi	2007	3	Unpublished
T.b. rhodesiense	TRPZ 26	Human	Zambia, Isoka	Unknown	Unknown	Godfrey et al. (1990)
T. evansi	RoTat 1.2	Water buffalo	Indonesia	1982	Unknown	Bajyana, Songa and Hamers (1988)
T.b. gambiense	MBA	Human	D.R. Congo, Bandundu	1974	9	Wéry et al. (1977)
T.b. gambiense	MONGO	Human	D.R. Congo, Bas-Congo	1968	8	Wéry et al. (1977)
T.b. gambiense	LiTat 1.3	Human	Côte d'Ivoire	1967	14	Van Meirvenne et al. (1977)
T.b. gambiense	MHOM/CD/INRB/2007/23A	Human	D.R.Congo, Mbuji-Mayi	2007	3	Unpublished
T.b. gambiense	MHOM/CD/INRB/2007/24B	Human	D.R.Congo, Mbuji-Mayi	2007	3	Unpublished
T.b. gambiense	MHOM/CD/INRB/2007/25B	Human	D.R.Congo, Mbuji-Mayi	2007	3	Unpublished
T.b. gambiense	MHOM/CD/INRB/2007/28	Human	D.R.Congo, Mbuji-Mayi	2007	3	Unpublished

PSG and centrifuged at 1500*g* for 15 min at room temperature. The parasite pellet was resuspended in 25 ml of PSG, centrifuged as above and the pellet was resuspended at a concentration of 5×10^6 cells ml^{-1} in 1 ml of HF containing 10% v/v glycerol for cryostabilisation (McCulloch et al., 2004). These cryostabilised strains could be used for culture start-up or rodent inoculation as described above.

2.6. Taxon identification

Parasite DNA from adapted cultured populations was extracted at peak parasitemia from 400 µl infected mouse blood using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. The identity of the trypanosome strains was confirmed via polymerase chain reaction (PCR) with *Trypanozoon* specific primers (Deborggraeve et al., in press) and *T.b. gambiense*, *T.b. rhodesiense* and *T. evansi* specific primers (Deborggraeve et al., 2008).

3. Results

3.1. Addition of methylcellulose

During the first round of *in vitro* experiments, we tested whether liquid or methylcellulose-containing HMI-9 based medium was appropriate for continuous axenic *in vitro* propagation of bloodstream form *Trypanozoon* strains. Eight of the 11 different *Trypanozoon* strains, one *T.b. brucei*, two *T.b rhodesiense*, one *T. evansi*, and four *T.b gambiense*, were inoculated in either HF or HFM. All eight strains showed initial growth in both media during primary culture for at least 24 h. Yet, the first sub passages made in HF were characterized by reduced growth rate, declining maximal cell density and abnormal cell morphology, usually resulting in cell culture cessation. After one or more sub passages from deteriorating populations, only two strains became adapted to HF: *T.b. brucei* AnTat 1.1^E and *T.b. rhodesiense* RUMPHI. An example of this adaptation process is given for *T.b. rhodesiense* RUMPHI in Fig. 1A. In HF, this strain showed a maximum cell density of 1×10^6 cells ml^{-1} and a doubling time of 13.5 ± 1.4 h.

In contrast to HF, from the first passage on, inoculation in HFM yielded exponentially growing populations as represented for *T.b. rhodesiense* RUMPHI in Fig. 1B. In HFM, this strain showed a higher maximum cell density of 4×10^6 cells ml^{-1} and lower doubling time of 9.8 ± 1.3 h. Thus, using the described *in vitro* adaptation protocols for established media, 2 of the 8 strains could be propagated over 10 consecutive sub passages in HF and 6 of the 8 strains could be continuously propagated in HFM (Table 3).

Among the 4 *T.b. gambiense* strains tested above, two, MBA and LiTat 1.3, were fully adapted to HFM, while two other, MHOM/CD/INRB/2007/23A and MONGO, failed in three independent attempts to grow in either HF or HFM.

3.2. Addition of methylcellulose and human serum

In the second round of *in vitro* experiments we used all seven *T.b. gambiense* strains to test whether, next to the addition of methylcellulose, the addition of human serum in the culture medium might help adaptation to *in vitro* culture (Table 1). An example is shown for the strain MHOM/CD/INRB/2007/23A, which was incubated in either HH or HHM. Inoculation at 5×10^4 or 5×10^5 cells ml^{-1} in HH medium yielded the same pattern as observed in HF and HFM medium with each next sub passage further deteriorating until cell culture cessation (Fig. 2A). In contrast, in HHM, exponential growth was observed in the primary culture and the same growth rate (12.4 ± 1.6 h doubling time) and maximum cell density (1.5×10^6 cells ml^{-1}) were retained during the 10 consecutive sub passages in continuous culture (Fig. 2B). Similar results were obtained for 4 other *T.b. gambiense* strains: one that previously failed to grow in HFM, MONGO, and 3 others that were isolated from the same HAT focus as MHOM/CD/INRB/2007/23A in the same year. These *T.b. gambiense* strains failed to grow when incubated in HH but could be continuously cultured in HHM for at least 10 consecutive sub passages. The *T.b. gambiense* strains LiTat 1.3 and MBA that could be maintained in HFM could also be continuously propagated in HHM (Table 3).

3.3. Taxonomic identity and infectivity

After 10 consecutive sub passages *in vitro* and after scale up and cryostabilisation, all strains retained their infectivity for mice giving rise to a detectable first peak parasitemia within 2–6 days after inoculation (data not shown). The species and subspecies identity of each trypanosome strain used in this study was confirmed by PCR (Table 4).

4. Discussion

The results obtained in this study demonstrate that an established method for continuous cultivation of pleomorphic *T.b. brucei* in methylcellulose can successfully be extended to *T.b. gambiense* only if human serum is included in the culture medium.

Only 2 of the 8 strains tested were readily adapted to HF medium, consisting of HMI-9 with 15% (v/v) fetal calf serum but without methylcellulose. One of these 2 strains was *T.b. brucei* AnTat 1.1^E, a low rodent passage variant of a widely used strain easy to grow in different culture media and rodent hosts. The other is *T.b. rhodesiense* RUMPHI, recently isolated from Malawi growing very rapidly in mice. As expected, none of the *T.b. gambiense* strains, although growing in mice and rats, became adapted in

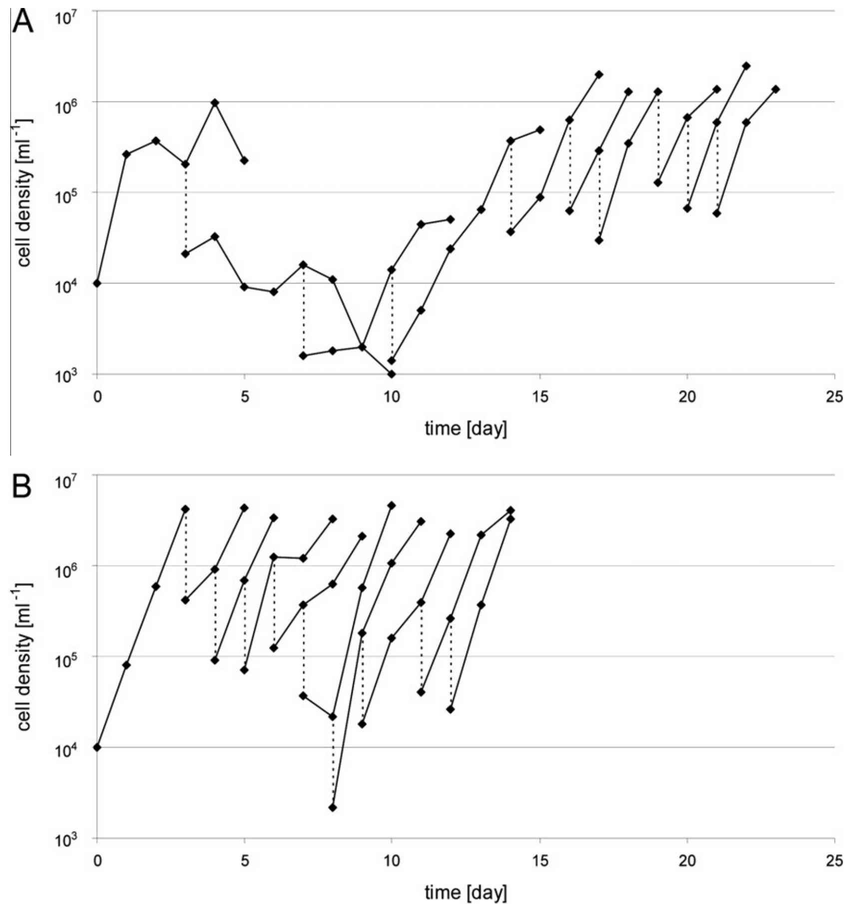

Fig. 1. *T.b. rhodesiense* in HF or in HFM. Growth curves of bloodstream form trypanosomes of *T.b. rhodesiense* RUMPHI, both derived lines were concurrently incubated *in vitro* at a start concentration of 1.0×10^4 cells ml^{-1} and propagated for 10 sub passages in either HF (panel A) or HFM (panel B) medium. Vertical dotted lines represent sub passages made during continuous culture using at least 80% fresh culture medium. Detection limit was 1.0×10^3 cells ml^{-1}.

Table 3
Overview of the adaptation of the trypanosome strains to the *in vitro* media.

Taxon	Strain	HF	HFM	HH	HHM
T.b. brucei	AnTat 1.1^E	Y	Y	n.d.	n.d.
T.b. rhodesiense	RUMPHI	Y	Y	n.d.	n.d.
T.b. rhodesiense	TRPZ 26	N	Y	n.d.	n.d.
T. evansi	RoTat 1.2	N	Y	n.d.	n.d.
T.b. gambiense	LiTat 1.3	N	Y	n.d.	Y
T.b. gambiense	MBA	N	Y	n.d.	Y
T.b. gambiense	MONGO	N	N	N	Y
T.b. gambiense	MHOM/CD/INRB/2007/23A	N	N	N	Y
T.b. gambiense	MHOM/CD/INRB/2007/24B	n.d.	n.d.	N	Y
T.b. gambiense	MHOM/CD/INRB/2007/25B	n.d.	n.d.	N	Y
T.b. gambiense	MHOM/CD/INRB/2007/28	n.d.	n.d.	N	Y

Y: continuous culture for at least 10 sub passages, N: not adapted. n.d.: not determined.

HF medium confirming the particular requirements of *T.b. gambiense* for its growth. Adding human serum to HF medium, becoming thus HH medium, was not successful for adaptation of 5 Congolese *T.b. gambiense* strains tested.

By adding 1.1% (w/v) methylcellulose to HF, 6 of the 8 tested strains became adapted to *in vitro* culture. Only 2 of the 4 inoculated *T.b. gambiense* strains could not be propagated in this medium. By combined addition of methylcellulose and human serum to HMI-9 supplemented with 15% (v/v) fetal calf serum, all 7 *T.b. gambiense* strains became adapted for continuous *in vitro* propagation in this new medium, called HHM.

The growth rates and maximum population densities observed in the different media are in line with previous studies on *in vitro* bloodstream form propagation in HMI-9 or MEM medium (Brun et al., 1981; Baltz et al., 1985; Hirumi and Hirumi, 1989). Yet, even with the addition of methylcellulose the number of harvested bloodstream forms from culture is still one or several orders of magnitude lower than what can be obtained from *in vitro* procyclic form cultures or from *in vivo* animal propagation.

In contrast with the liquid axenic HMI-9 or feeder layer cell MEM culture method, the original diversity within the inoculated trypanosome population might be better preserved when methylcellulose is included in the medium. Indeed, medium with methylcellulose prevents part of the population dying off during the adaptation to the medium and thus prevents or at least diminishes selection during primary *in vitro* culture adaptation, a phenomenon that can have uncontrolled consequences on downstream characterization of the populations, e.g. genotyping, mixed infections, drug sensitivity profile etc. (Baltz et al., 1985; Hirumi and Hirumi, 1989; Vassella and Boshart, 1996).

It remains to be proven if HFM or HHM medium can be applied for direct isolation of metacyclics from tsetse saliva or of bloodstream form trypomastigotes from human blood, lymph or cerebrospinal fluid as was reported by others but for primary cultures on feeder cell layers and with long adaptation periods (Brun et al., 1979, 1981, 1984; Truc et al., 2004; Giroud et al., 2009). Furthermore, it might be interesting to investigate if methylcellulose might also be of use in culture media for *T. vivax* or *T. congolense*, two animal pathogens that are very difficult to adapt

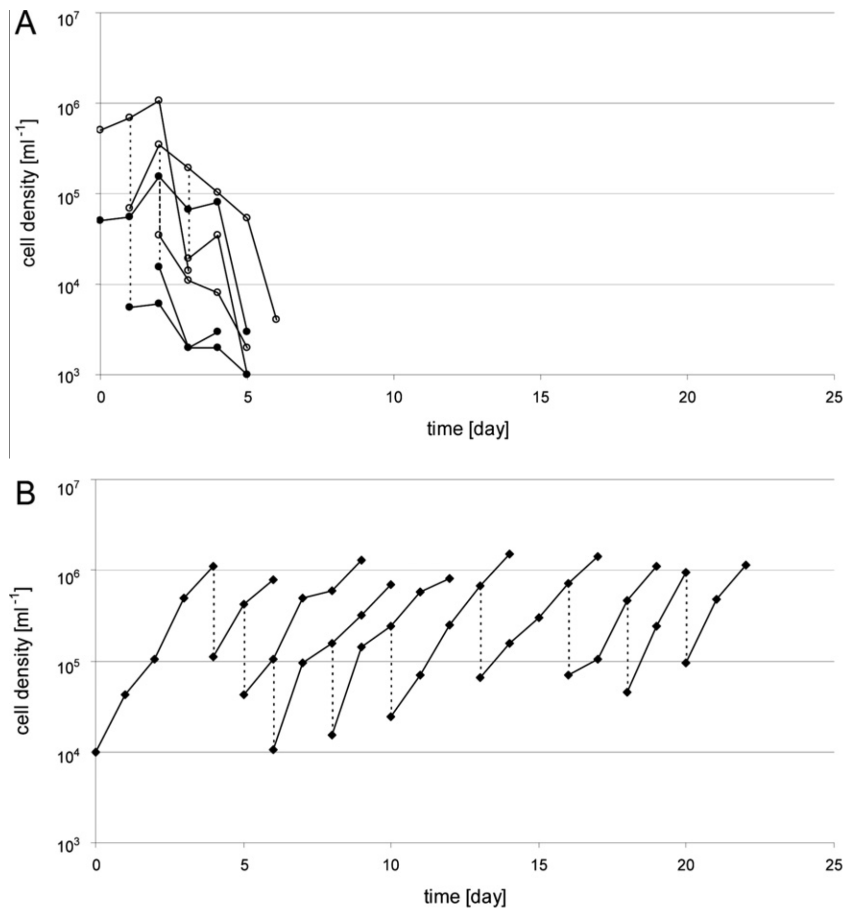

Fig. 2. *T.b. gambiense* in HH or in HHM. Growth curves of bloodstream form trypanosomes of *T.b. gambiense* MHOM/CD/INRB/2007/23A, both derived lines were concurrently incubated *in vitro* in HH (panel A) and HHM (panel B). Vertical dotted lines represent sub passages made using at least 80% fresh medium. Panel A: primary cultures in HH started with 5.0×10^4 and with 5.0×10^5 cells ml^{-1}. Panel B: primary culture in HHM started with 1.0×10^4 cells ml^{-1}. Detection limit was 1.0×10^3 cells ml^{-1}.

Table 4
Results of taxon specific PCR and interpretation.

Strain	M18S[a]	TgsGP[b]	SRA[c]	RoTat 1.2[d]	Interpretation
AnTat 1.1[E]	P	N	N	N	*T.b. brucei*
RUMPHI	P	N	P	N	*T.b. rhodesiense*
TRPZ 26	P	N	P	N	*T.b. rhodesiense*
RoTat 1.2	P	N	N	P	*T. evansi*
LiTat 1.3	P	P	N	N	*T.b. gambiense*
MBA	P	P	N	N	*T.b. gambiense*
MONGO	P	P	N	N	*T.b. gambiense*
MHOM/CD/INRB/2007/23A	P	P	N	N	*T.b. gambiense*
MHOM/CD/INRB/2007/24B	P	P	N	N	*T.b. gambiense*
MHOM/CD/INRB/2007/25B	P	P	N	N	*T.b. gambiense*
MHOM/CD/INRB/2007/28	P	P	N	N	*T.b. gambiense*

P: positive, N: negative.
[a] M18S: *Trypanozoon* specific PCR targeting the 18S ribosome subunit gene.
[b] TgsGP: *T.b. gambiense* specific PCR targeting the glycoprotein 19 gene.
[c] SRA: *T.b. rhodesiense* specific PCR targeting the serum resistance associated gene.
[d] RoTat 1.2: *T. evansi* specific PCR targeting the rode *Trypanosoma* antigen type 1.2 gene.

to rodents and culture media (Brun and Moloo, 1982; Zweygarth et al., 1991; Hirumi and Hirumi, 1991).

5. Conclusions

In summary, we can confirm that an improved *in vitro* medium for continuous propagation of bloodstream form non-*gambiense* *Trypanozoon* strains is prepared by supplementing methylcellulose to HMI-9 based medium. However, for the continuous axenic *in vitro* propagation of freshly isolated *T.b. gambiense* strains, the addition of human serum to methylcellulose containing HMI-9 is critical.

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