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Isolation, Cloning and Cultivation of *Trypanosoma danilewskyi* strain FCc 1 in Different Culture Media*

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Abstract.- *Trypanosoma danilewskyi* strain FCc 1 was isolated from a naturally infected carp, *Cyprinus carpio*. The parasites were purified from infected blood through DEAE 52 cellulose column and inoculated in naïve-cloned carps for *in vivo* maintenance. At the peak of infection from subpassage 2, the trypanosomes (blood stream forms) were cloned (single flagellate/fish) in naïve-cloned carps. The cloned *T. danilewskyi* was given a strain name as *Trypanosoma danilewskyi* strain FCc 1. This strain was cultivated in different culture media. Among culture media used for culture initiation, Tobie's medium supported trypanosome growth significantly. While the media used for regular cultivation of trypanosomes for mass production, RPMI 1640 was excellent for the cultivation of *T. danilewskyi* strain FCc 1.

Key words: Flagellates cultivation, culture media, naïve-cloned carp, RPMI 1640.

INTRODUCTION

During last few decades' excellent progress has been made in the *in vitro* cultivation of different species of African trypanosomes, agent of trypanosomiasis in human and other mammals. *In vitro* culture of freshwater fish trypanosome has also been documented. Cuningham *et al.* (1981) successfully cultured metacyclic forms, while blood srteam forms were cultured by Brun *et al.* (1981), Zweygarth *et al.* (1989) and Hirumi and Hirumi (1991).

In vitro culture of freshwater fish trypanosome was also described at the beginning of this century. Early attempts with various modifications of blood agar media supported the growth but their subcultures were not established. *Trypanosoma danilewskyi* (strains MS and MA) isolated from carp (*Cyprinus carpio*) were cultured in MEM medium with 10% fetal bovine serum (FBS) and a continuous growth was obtained at 25°C (Smolikova *et al.*, 1977).

Lom and Suchankova (1974) used biphasic blood agar media for the culture of freshwater trypanosome species like *T. danilewskyi* isolated from carp (*Cyprinus carpio*), *T. percae* from perch (*Perca fluviatilis*) and *T. remaki* from pike (*Esox lucius*). Smolikova *et al.* (1977) cultivated *T. danilewskyi* on modified Eagle's MEM medium with 10% fetal calf serum (FCS) at 25°C and enriched the medium with hemin (2 mg ml^{-1}) for better results. The strain he used, was previously cultured on biphasic blood agar medium during culture initiation. Nihukova (1977) grew *T. danilewskyi* on biphasic SNB-9 medium at 25°C. Zajicek and Peckova (1990) cultured different species of trypanosomes isolated from freshwater fishes on SNB-9 medium that was replaced by L4 NHS medium in subsequent subculture. *Trypanosoma granulosum* from eel, *Anguilla anguilla*, was cultivated on horse blood-nutrient agar with an overlay of Ringer's solution at 18-22°C (Davies *et al.*, 1992).

The present study was undertaken to cultivate the trypanosomes isolated from naturally infected carp (*Cyprinus carpio*) *in vivo* (in juvenile cloned carp) and *in vitro* in different culture media.

MATERIALS AND METHODS

Big carps (±3 kg each) were purchased from a commercial fish farm and kept in aquaria at 20°C in a flow through system. All fish were diagnosed for trypanosome infection and the fish found infected were separated. For *in vivo* maintenance trypanosome were inoculated in 8 months old laboratory bred naïve-cloned carps (12.6±3.2 cm in length), purchased from the Experimental Fish Facility, Zodiac Department, Agricultural University, Wageningen, The Netherlands. The blood from the infected fish was taken from caudal vein with a sterile syringe. To avoid bacterial contamination, the skin was wiped off first with tissue paper and then with sterilized distilled water.

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Trypanosomes were purified from blood cells by using DEAE 52 cellulose (DE 52 preswollen) in a polypropylene column (Lanham and Godfrey, 1970). Elutes were washed by centrifugation in phosphate buffered saline with glucose (PSG: pH 8, conductivity 7.5mho) at 20°C and used for *in vivo* sub passages (*in vivo* maintenance). The primary isolate was given a code name according to World Health Organization recommendation as FCc/BE/90/KULZO=1 (Fish *Cyprinus carpio*/Belgium/1990/Katholieke Universiteit Leuven, Zoological Institute = 1) which was abbreviated as FCc 1. Infected blood with parasitemia $6.3x10^6$ trypanosomes ml^{-1}, was used for cultivation in culture media. All experimental fish were fed an equivalent of 3% body weight daily.

Statistical analysis of the data was done using SAS (Statsoft Inc., 1989) computer package. Bonferroni (Dunn) t-test was also applied.

In vivo *maintenance*

Two groups (A and B) of naïve-cloned carps each containing 10 fish, were maintained in glass aquaria at 20°C for two weeks in flow through system prior to infection. Fish from group A were intraperitoneally inoculated with 0.1 ml trypanosome suspension ($6.3x10^5$ trypanosomes) and group B received same amount of PSG (filter sterilized) as a control. Development of infection was monitored by weekly examination of blood from each fish in both infected and control groups until 63 days post inoculation. At each peak, trypanosomes were inoculated (subpassage) in 5 new carps to maintain the strain *in vivo*. The parasitemia was estimated by rapid Matching Method (Herbert and Lumsden, 1976).

In vivo *cloning*

Infected blood was taken at the peak of infection from an infected fish during *in vivo* maintenance (subpassage 2). One 1 infected blood was serially diluted with 1 ml PSG in 96 microtiter plate (Nunc) until a single trypanosome was found in final dilution. The content of each well was examined under phase contrast microscope. An inoculum of a single trypanosome per fish was given intraperitoneally to 5 naïve-cloned carps. Parasitemia was checked weekly and estimated by Hematocrit Centrifugation Technique (HCT) (Woo, 1969) for very low parasitemia and rapid Matching Method for high parasitemia wherever necessary.

In vitro *cultivation*

For *in vitro* cultivation five culture media, commonly used for cultivation of African trypanosomes and fish trypanosomes, were selected. Three of them (Tobie's medium (Tobie *et al.*, 1950), SNB-9 medium (Taylor and Baker, 1987) and Brain Heart Infusion (BHI) with rabbit blood, were used for culture initiation. Two media (GLSH-DCA (Le Ray, 1975) and RPMI 1640 (w/L-glutamine, w/o NaHCO3, Gibco, Life Technologies, UK) were used for regular cultivation.

For *in vitro* culture initiation, bloodstream forms of strain FCc 1 were obtained from fish at peak of cloning infections. Four tubes from three media (culture initiation media) were inoculated with 0.1 ml infected blood ($7.8x10^5$ trypanosomes). All tubes were incubated at 20°C. Locke-Krebs overlay (Le Ray, 1975) with MEM vitamin solution (Gibco) (Ahmed, 1994), 0.3 ml was added to each tube of Tobie's medium while SNB-9 medium without agar was used as an overlay in SNB-9 agar medium. Parasite propagation was checked after three days post inoculation and weekly thereafter.

For *in vitro* regular cultivation, four tubes from each culture medium were inoculated with trypanosomes adapted in Tobie's medium (0.1 ml culture containing $3x10^5$ flagellates). All tubes were incubated at 20°C and parasitemia was checked daily.

RESULTS

In vivo *maintenance*

Trypanosomes were found in 7 fish out of 10, in group A on day 4 post inoculation (*p.i*) and all fish were positive on day 7 *p.i.* The parasitemia was monitored weekly and a peak of $3.98x10^6$ trypanosomes ml^{-1} was obtained on day 42 *p.i.* (Fig. 1). No fish from group B was found infected.

In vivo *cloning*

Out of 5 fish, two fish were positive on day 7 and all of them were positive on day 10 *p.i.* Parasitemia was very low in the beginning but increased rapidly on day 28 to 35 and a peak of $1x10^6$ trypanosomes ml^{-1} was obtained on day 56 *p.i.* (Fig. 2). During the successive subpassages, the infection developed in the same way as *in vivo* maintenance experiments.

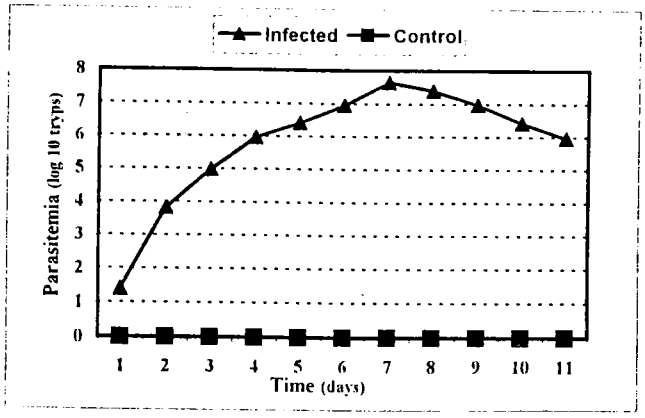

Fig. 1. Development of parasitemia during the infection of *T. danilewskyi* strain (blood stream forms) in naïve-cloned carps, *Cyprinus carpio*.

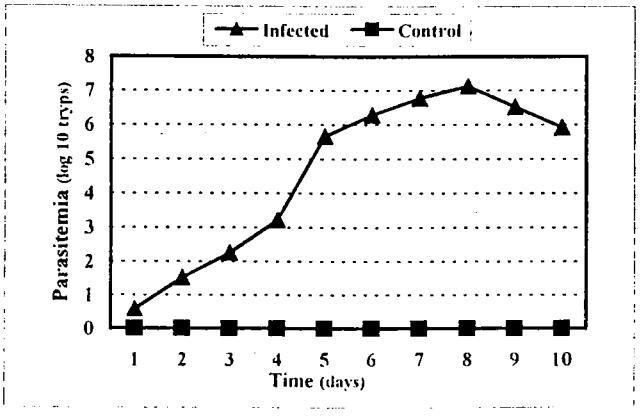

Fig. 2. Development of parasitemia during cloning of *T. danilewskyi* strain FCc 1 in naïve-cloned carps, *Cyprinus carpio*.

In vitro *cultivation*

Culture initiation

Tubes from all media were positive with live trypanosomes from day 1 to 10 post inoculation. Tobie's medium supported growth until 7th subculture and dividing trypanosomes were found. Metatrypanosomes (blood stream form) dominated in the trypanosome population and dividing cells producing two unequal, epimastigotes and trypomastigotes (mostly) or equal, trypomastigotes (rarely) were observed. Very small colonies (3-7 trypanosomes/colony) were seen in 6th and 7th subcultures.

Trypanosomes remained alive in SNB-9 medium until 4th subculture but the number of trypanosomes was low and finally decreased to zero. Flagellate division was rare, only stumpy forms were sometimes observed. No trypanosomes were found in subculture 5 or until day 20 *p.i.* in the same subculture.

Live trypanosomes were found in BHI but no cell division was observed until subculture 5. Only slender long trypomastigote forms dominated. Later, trypanosome number started decreasing and trypomastigotes disappeared in the 7th subculture.

Regular cultivation

The responses of culture media for culture initiation as well as cultivation are given in Table I. In Tobie's medium, until subculture 3, trypomastigote forms predominate. Dividing trypomastigotes were unequal (epimastigotes and trypomastigotes) and common in the supernatant of the medium. In subsequent subcultures, the dividing stage predominated (although some metatrypanosome forms were present) and a good growth of $4x10^6$ tryps. ml^{-1} was obtained until subculture 10 (Fig. 3). Parasite colonies were also present. The growth was lowered later but when inoculation from Tobie's medium was made in RPMI 1640, rapid growth ($7.9x10^6$ tryps. ml^{-1}) was obtained on day 6 (Fig. 4). In Tobie's medium, the trypanosome number significantly ($P<0.05$) increased from day 3 to 8 as compared with day 1. The parasite growth was significantly ($P<0.05$) higher from the growth obtained in GLSH-DCA from day 5 to 8. Tobie's medium was found the best for culture initiation as well as cultivation of *T. danilewskyi* strain FCc 1.

The GLSH-DCA medium supported growth until 8th subculture but the growth ($7.5x10^5$ tryps. ml^{-1}) was lower and very few dividing cells were seen. Few colonies (3-5 flagellates) were found. In GLSH-DCA, the trypanosome number significantly ($P<0.05$) increased from day 4 to 8 as compared with day 1. The parasite growth was significantly ($P<0.05$) lower than the growth obtained in Tobie's medium from day 5 to 8. When culture from GLSH-DCA was inoculated in RPMI 1640, rapid growth ($7.5x10^6$ tryps. ml^{-1}) was obtained on day 7 *p.i.*

When RPMI 1640 was inoculated with adapted trypanosomes from Tobie's medium, a significant ($P<0.05$) growth ($7.9x10^6$ tryps. ml^{-1}) was observed but the growth of non-adapted cultures was not

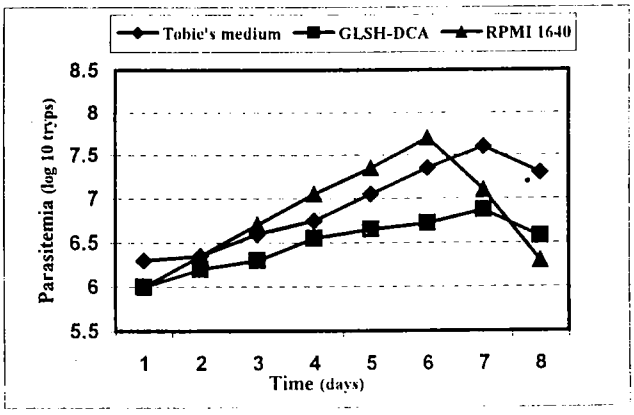

Fig. 3. The growth of *T. danilewskyi* strain FCc 1 in different culture media.

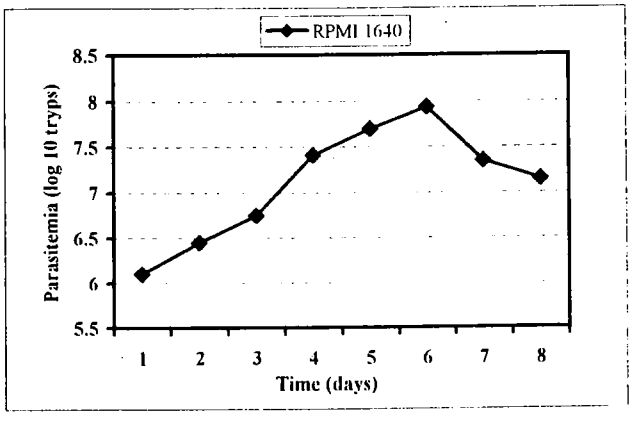

Fig. 4. The growth of *T. danilewskyi* strain FCc 1 in RPMI 1640 when inoculated trypanosomes from Tobie's medium.

satisfactory. Trypomastigotes were dividing into unequal, epimastigote and trypomastigote, and binary fission type of division was predominant. Colony formation was also observed. In RPMI 1640, the trypanosome number significantly ($P<0.05$) increased from day 3 to 8 as compared with day 1. The parasite growth was significantly ($P<0.05$) higher as compared to the growth obtained in GLSH-DCA on day 3 to 6 but decreased on day 6 to 7 as compared to Tobie's medium (Table II).

DISCUSSION

The leech vectors transmit infection in fishes in natural conditions. In laboratory conditions, laboratory bred experimental animals can be infected by vector or syringe (syringe passage); such infections develop in the same way as in the natural conditions in the recipient hosts. The host's immune responses activate against the trypanosomes and are eliminated after the peak of infection. During the experimental infections of cloned *T. danilewskyi* strain FCc 1 in naïve-cloned carps at 20°C, the infection developed rapidly and the peak was obtained from day 35 to 42 *p.i.*

During the cloning, the infection developed slowly in the beginning and the peak was obtained on 56 days *p.i.* The delayed peak of infection was due to the smaller amount of inoculum. The similar number of parasites at the peak of infection is in accordance with the results of Lom (1979); according to him the number of flagellates inoculated are not correlated with their number obtained at the peak of infection.

The blood stream forms undergo multiplicative divisions in the culture media. Biphasic media, *i.e.* blood agar with overlay, are mostly used for culture initiation and give better results. The culture initiation of *T. danilewskyi* strain FCc 1 in BHI with rabbit blood) was not successful, while in Tobie's medium trypanosome propagation was significant because of Locke-Krebs overlay containing many organic and inorganic salts and MEM vitamin solution.

Trypanosome multiplication and growth was better in monophasic media, when only adapted trypanosomes in Tobie's medium, were inoculated in synthetic media (RPMI 1640 and GLSH-DCA). The yield of monophasic media's was always higher (Cunningham and Honigberg, 1977) than the biphasic media. The yield of *T. danilewskyi* strain FCc 1 in Tobie's medium (1 ml overlay) was $4x10^6$ trypanosomes/tube (weekly) while a similar inoculum produced $1.58x10^7$ flagellates/tube (2 ml) in RPMI medium within the same time. The yield of GLSH-DCA was, however, lower than Tobie's but shifting of inoculum from one medium (GLSH-DCA) to other like RPMI 1640, resulted in a rapid growth and a yield of $1.5x10^7$ trypanosomes/tube/week was obtained. The *T. danilewskyi* strain FCc 1 from *in vivo* maintenance, cloning and different cultures were cryopreserved at Cryobank, Institute of Tropical Medicine and Hygiene, Antwerpen, Belgium.

Table I.- ***In vitro*** **culture initiation and cultivation of** ***T. danilewskyi*** **strain FCc 1 in different culture media at 20 °C. Live trypanosomes present or absent (+/ or -/) and dividing forms present or absent (/+ or /-).**

Culture media	Time (days)							
	1	2	3	4	5	6	7	8
Culture initiation								
Tobie's medium	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+
SNB-9	+/-	+/-	+/+	+/-	+/-	-/-	-/-	-/-
BHI	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-
Cultivation								
Tobie's medium	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+
GLSH-DCA	+/-	+/-	+/-	+/+	+/+	+/+	+/-	+/-
RPMI 1640	+/-	+/-	+/-	+/+	+/+	+/+	+/-	+/-

Table II.- **The growth of** ***T. danilewskyi*** **strain FCo 1 in different culture media at 20 °C. Data are expressed as log 10 tryps/ml. a and b indicate the significant difference (P<0.05) from day 1 and x indicates a significant difference (P< 0.01) from RPMI 1640 at different time.**

Culture media	Time (days)							
	1	2	3	4	5	6	7	8
Tobie's	6.3±0.18	6.35±0.23	6.6±0.26a	6.75±0.24a	7.05±0.34b	7.35±0.13b	7.6±0.25bx	7.3±0.22bx
GLSH-DCA	6.0±0.12	6.2±0.25	6.3±0.22x	6.55±0.27ax	6.65±0.21ax	6.72±0.23bx	6.87±0.16bx	6.57±0.21ax
RPMI 1640	6.0±0.17	6.35±0.33	6.7±0.21ax	7.05±0.12bx	7.35±0.25bx	7.7±0.17bx	7.1±0.21bx	6.3±0.19x
when adapted in Tobie's medium and cultivated in								
RPMI 1640	6.1±0.21	6.45±0.21a	6.75±0.31a	7.41±0.12b	7.70±0.25b	7.94±0.2b	7.35±0.25bx	7.15±0.13bx

mean ± SEM

Only Tobie's medium seemed good for isolation, adaptation and cultivation of *T. danilewskyi* strain FCc 1. Most flagellate parasites require glucose as a source of carbon and exogenous energy for multiplication (Gutteridge and Coombs, 1977; Khler and Voigt, 1988). *Trypanosoma brucei* die rapidly in the absence of glucose in the culture medium (Ghiotto *et al.*, 1979) and *Cryptobia salmositica* divide rapidly utilizing glucose in the medium supplemented with FCS, containing sufficient amount of glucose and minerals. Similarly *T. danilewskyi* strain FCc 1 grew in Tobie's medium with an overlay containing enough glucose while other initiation media (SNB-9 and BHI) did not contain sufficient amount of glucose.

The metabolic waste products of protozoan are carbon dioxide, pyruvate, urea and ammonia (Larsen *et al.*, 1988). These metabolites are toxic for parasites when they accumulate in the media and are responsible for the lysis of parasites and

formation of round forms (Li and Woo, 1991) in the old cultures. The increase in the percentage of lysed cells and rounded forms is due to depletion of nutrients and accumulation of toxic metabolites in the culture media. These round forms are mostly without nucleus or kinetoplast and are devoid of the dividing capabilities.

REFERENCES

AHMED, M.S., 1994. *Trypanosomiasis in common carp*, Cyprinus carpio L. Ph.D. thesis, Department of Biology, Katholieke Universiteit, Leuven, Belgium.

BRUN, R., JENNI, L., SCHONENBERGER, M AND SCHELL, K. F., 1981. *In vitro* cultivation of blood stream forms of *Trypanosoma brucei*, *T. rhodesiense* and *T. gambiense*. *J. Protozool.*, **28**: 470-479.

CUNNINGHAM, I. AND HONIGBERG, B.M., 1977. Infectivity reacquisition by *Trypanosoma brucei* brucei cultivated with tsetse salivary glands. *Science*, **197**: 279-1282.

CUNNINGHAM, I., HONIGBERG, B.M. AND TAYLOR, A.M., 1981. Infectivity of monophasic and pleomorphic *Trypanosoma brucei brucei* stocks cultivated at 28 ° C with various tsetse fly tissues. *J. Parasitol.*, **67**: 391-397.

DAVIES, A.J., MASTRI, C., ARIYANAYANGAM, M.R., SAPPAL, G.K. AND WILKIN, T.A., 1992. Simple methods for culturing and preserving *Trypanosoma granulosum* Lav. and Mes. from European eels (Anguilla anguilla). *Bull. Eur. Assoc. Fish Pathol.*, **12**: 8-10.

GHIOTTO, V., BRUN, R., JENNI, L. AND HECKER, H., 1979. *Trypanosoma brucei*: Morphometric changes and loss of infectivity during transformation of blood stream forms to procyclic culture forms *in vitro*. *Exp. Parasitol.*, **48**: 447-456.

GUTTERIDGE, W.E. AND COOMBS, G.H., 1977. *Biochemistry of parasitic protozoa*. University Park Press, Baltimore, Maryland, 172.

HERBERT, W.J. AND LUMSDEN, W.H.R., 1976. *Trypanosoma brucei*: A rapid "Matching" method for estimating the host's parasitemia. *Exp. Parasitol.*, **40**: 427-439.

HIRUMI, H. AND HIRUMI, K., 1991. *In vitro* cultivation of blood stream *Trypanosoma congolence* bloodstream forms in the absence of feeder cell layer. *Parasitology*, **102**: 225-236.

KOHLER, P. AND VOIGT, W. P., 1988. Nutrition and metabolism. In: *Parasitology in focus* (ed. H. Mehlhorn), pp. 412-453. Springer-verlag, Berlin, Heidelberg.

LANHAM, S.M. AND GODFREY, D.G., 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Exp. Parasitol.*, **28**: 521-534.

LARSEN, J., SVENSMARK, B. AND NILSSON, J.R., 1988. Variation in the growth medium during the culture cycle of Tetrahymena with special reference to ammonia (NH_3), ammonium ($NH+_4$) and pH. *J. Protozool.*, **35**: 541-546.

LE RAY, D., 1975. Structures antigeniques de *Trypanosoma brucei* (Protozoa, Kinetiplastida). *Ann. Soc. Belg. Med. Trop.*, **55**: 159.

LI, S. AND WOO, P.T.K., 1991. *In vitro* effects of fetal bovine serum and glucose on multiplication of *Cryptobia salmositica*. *J. Parasitol.*, **77**: 151-155.

LOM, J., 1979. Trypanosomes and Trypanoplasms of fish. In: *Biology of Kinetoplastida* (eds.W. H.R. Lumsden and D.A. Evans), vol. 2, pp. 269-302. Academic Press, London.

LOM, J. AND SUCHANKOVA, E., 1974. Comments on the life cycle of *Trypanosoma danilewskyi*. Proceedings of the III International Congress on Parasitology, vol. 1, pp. 66-67. Facta Publication, Munich.

NOHYNKOVA, E., 1977. *Trypanosoma danilewskyi*: *In vitro* culture of dyskinetoplastic forms. *J. Protozool.*, **24**: 47 A.

SAS INSTITUTE INC, 1989. SAS/STAT User Guide, Version6, 4th eds., vol. 2. SAS Institute Inc., Cary, North Carolina, USA.

SMOLIKOVA, V., LOM, J. AND SUCHANKOVA, E., 1977. Growth of carp trypanosome *T. danilewskyi* in fish tissue culture. *J. Protozool.*, **24**: 52 A.

TAYLOR, A.E.R. AND BAKER, J.R., 1987. In vitro *methods for parasite cultivation*. Academic Press, London.

TOBIE, E.J., VAN BRAND, T. AND MEHLMAN, B., 1950. Cultural and physiological observation on *Trypanosoma rhodesiense* and *Trypanosoma gambiense*. *J. Parasitol.*, **36**: 48-54.

WOO, P.T.K., 1969. The hematocrit centrifuge technique for the detection of trypanosomes in blood. *Can. J. Zool.*, **47**: 921-923.

ZAJICEK, P. AND PECKOVA, H., 1990. The interaction of fish trypanosome culture forms with some lectins. *Folia Parasitol.*, **37**: 1-8.

ZWEYGARTH, E., KAMINSKY, R. AND CHERUIYOT, J. K., 1989. A simple and rapid method to initiate *Trypanosoma brucei* brucei and *T. brucei evansi* blood stream form cultures. *Acta Trop.*, **46**: 205-206.

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