The Journal of Animal & Plant Sciences, 24(1): 2014, Page: 149-153
ISSN: 1018-7081

SIMPLE AND RAPID CULTIVATION OF *TRYPANOSOMA DANILEWSKYI* STRAIN FCC-1 IN MONOPHASIC LIQUID CULTURE MEDIUM

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ANSTRACT

Trypanosoma danilewskyi strain F Cc-1 was isolated from the blood of a laboratory infected juvenile common carp. Trypanosomes (blood stream forms) were first adapted to culture conditions in Tobie's medium. Ten sterilized glass culture tubes each containing 2 ml of Tobie's medium solidified in slants and covered with 1 ml Locke-Krebs overlay, were inoculated with 0.1 ml of infected blood containing 1x 10^5 live trypanosomes and incubated at 20º C. Tobie's medium supported growth and trypanosomes were well adapted to culture conditions because small colonies were seen in 6th and 7th subculture. The adapted trypanosomes were re-inoculated in a monophasic liquid culture medium (L4 NHS). Ten sterilized caped glass culture tubes, each containing 3 ml of medium, were inoculated with 0.1 ml culture containing 3 x 10^5 flagellates and incubated at 20º C. Trypanosome propagation / multiplication were examined on day 3 p.i. and daily later on and their number ml^{-1} was estimated by the Rapid Matching Method. Trypanosomes growth obtained in L4 NHS culture medium on day 7-8 was 5.0 ± 0.56 x 10^6 live trypanosomes ml^{-1}. The trypanosome number significantly ($P<0.05$) increased from day 3 to 8 as compared to day 1. Flagellate numbers remained high until day 10 but decreased from day 12-15 p.i. L4 NHS, mono-phasic culture medium supported growth of *T. danilewskyi* strain FCc-1 (culture forms) significantly high ($P<0.01$) at 20º C and found the best for rapid fish trypanosome culture.

Key words: rapid culture, monophasic medium, strain F Cc-1, L4NHS, blood flagellates, fish

INTRODUCTION

Excellent progress has been made in the *in vitro* cultivation of different species of African trypanosomes, agents of trypanosomiasis in human and other mammals. *In vitro* culture of freshwater fish trypanosome is also documented. *Trypanosoma danilewskyi* (strains MS and MA) isolated from common carp (*Cyprinus carpio*) were cultured in MEM medium with 10 % fetal bovine serum (FBS) and continuous growth was obtained at 25°C (Nohynkova, 1977, Smolikova, 1977). Later, he cultivated the same strains on modified Eagle's MEM medium with 10 % fetal calf serum (FCS) and enriched medium with hemin, 2 mg ml^{-1}, for better results. Ahmed (1994) and Ahmed *et al*., (2001) efficiently cultured *T. danilewskyi* strain F Cc-1 in Tobie's, GLSH-DCA and RPMI 1640 media at 20 °C.

Marine fish trypanosome cultivation has also been reported. The best cultivation was obtained on liquid and semi-liquid culture media (brain heart infusion agar with blood and nutrient broth with blood). These culture media supported growth of *Trypanosoma triglae senegalensis* but failed to support the growth of *Trypanosoma cephalacenthi* and *Trypanosoma shaeroidis* (Lom, 1979). Jones and Woo (1991) cultivated *Trypanosoma catostomi* and *T. phaleri* from North American fishes in Eagle's basal medium (BME) with Hank's salt and L-glutamine, supplemented with 10 % fetal bovine serum (FBS) at 20-22°C.

In the present study a mono-phasic liquid culture medium (L4NHS) was tried out to develop a simple and efficient method for the growth of *T. danilewskyi* strain F Cc-1 under laboratory conditions.

MATERIALS AND METHODS

Adaptation of strain FCc-1 in culture condition: Blood stream form (BSF) of *Trypanosoma danilewskyi* strain FCc-1 was isolated from a laboratory infected juvenile common carp which were maintained in the juvenile common carp by syringe passage until 12th sub-passage (Ahmed, 1994; Ahmed *et al*., 2001). Trypanosomes were adapted to culture conditions in Tobie's medium (Tobie *et al*., 1950) (Table 1). Ten sterilized glass culture tubes each containing 2 ml of Tobie's medium solidified in slants and covered with 1 ml Locke-Krebs solution as an overlay (Le Ray, 1975), were inoculated with 0.1 ml infected blood containing approximately 1x 10^5 live trypanosomes and incubated at 20º C. Trypanosome propagation was examined on day 3 p.i. (post inoculation), daily later on and their counts ml^{-1} were estimated by Rapid Matching Method (Herbert and Lumsden, 1976). Subcultures were made weekly or whenever necessary from five culture tubes while other

five culture tubes were maintained for follow-up of further growth.

Cultivation of strain FCc-1 in monophasic culture medium: Adapted trypanosomes from Tobie's medium were cultured in freshly prepared monophasic liquid culture medium, L4 NHS (Baker *et al*., 1976) (Table 2). Ten sterilized caped glass culture tubes each containing 3 ml of medium, were inoculated with 0.1 ml trypanosome culture containing 3 x 10^5 live adapted trypanosomes and incubated at 20º C. Trypanosome propagation was monitored as mentioned in previous section. Subcultures were also made where necessary. The history of *T. danilewskyi* strain FCc-1 (BSF & CF) is detailed in figure 1.

Cryopreservation of T. danilewskyi strain FCc-1: Seven days old culture of *T. danilewskyi* strain FCc-1 from L4 NHS culture medium was washed and re-suspended in freshly prepared L4 NHS medium and then added 1/3 volume of 5 % DMSO in PBS (Hirumi *et al*., 1977, Hirumi *et al*., 1980, Davies *et al*., 1992). One ml of this mixture was dispensed in sterilized glass ampoules. Ampoules were sealed by flame and cryopreserved at Cryobank, Institute of Tropical Medicine and Hygiene, Antwerp, Belgium.

RESULTS

Adaptation of trypanosomes to culture conditions: Tobie's medium supported growth until 7th subculture and live and dividing trypanosomes were found in subsequent subcultures (Table 3). Metatrypanosomes (blood stream form) dominated among trypanosome population until 3rd subculture. But later a binary fission type of division was observed producing two unequal epimastigotes and trypomastigotes (mostly) or equal trypomastigotes (rarely). Very small colony formation (3-7 cells/colony) was seen in 6th and 7th subculture (Figure 2).

Cultivation and growth: Growth in monophasic culture medium (L4 NHS) was excellent and the maximum number of trypanosomes (5.0 ± 0.56 x 106 trypanosomes ml^{-1}) was obtained in 7 to 8 day old culture tubes (Figure 3). The trypanosome number significantly ($P<0.05$) increased from day 3 to 8 as compared with day 1. Many dividing trypomastigotes were seen in 5th sub-cultures. Metatrypanosome forms predominate and an aggregation of a few trypanosomes (5-9 cells) was making small colonies which ultimately grew bigger on day 7 of the culture. Flagellate numbers remained high until day 10 in the same culture but decreased from day 12-15 p.i. Trypomastigotes and metatrypanosomes (long slender & stumpy forms) were moving slowly and very few dividing forms were seen after day 15. Culture remained positive until day 20 with very low number of flagellates and became negative on day 25. The subcultures were made weekly while previous culture tubes were maintained to follow further growth. This mono-phasic culture medium supported growth of *T. danilewskyi* strain FCc-1 significantly high ($P<0.05$) at 20º C and found the best for freshwater fish trypanosome cultivation.

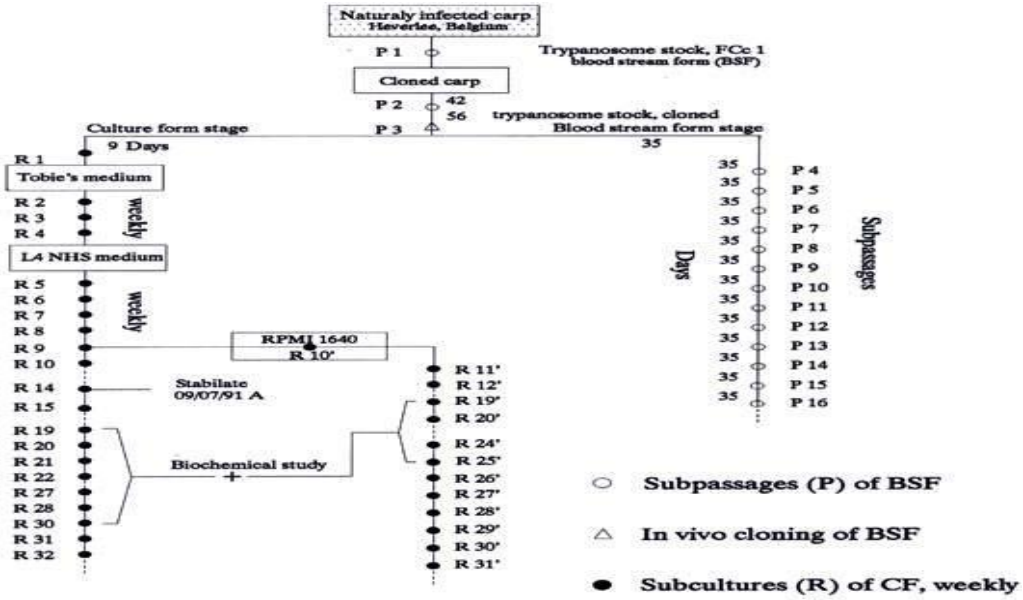

Figure 1: The pedigree diagram of *T. danilewskyi* strain FCc-1 (BSF & CF) isolated from a naturally infected common carp (*Cyprinus carpio*), cloned and maintained in juvenile common carp until 12th sub-passage. Blood stream forms were adapted on Tobie's medium and then cultivated in L4 NHS from 4th sub-culture.

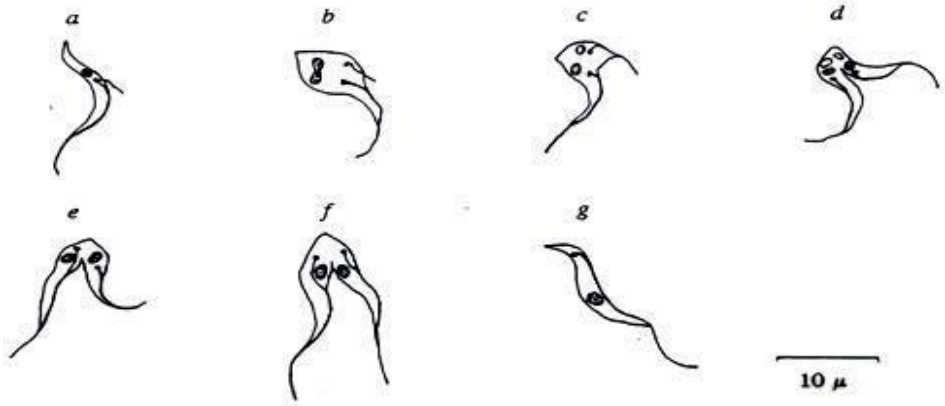

Figure 2: Various morphological types of *T. danilewskyi* strain FCc 1 from Tobie's culture medium: (a) dividing epimastigotes with a new flagellum; (b-e) unequal epimastigotes division producing epimastigotes and trypomastigotes; (f) dividing trypomastigotes producing equal trypomastigotes; (g) trypomastigotes.

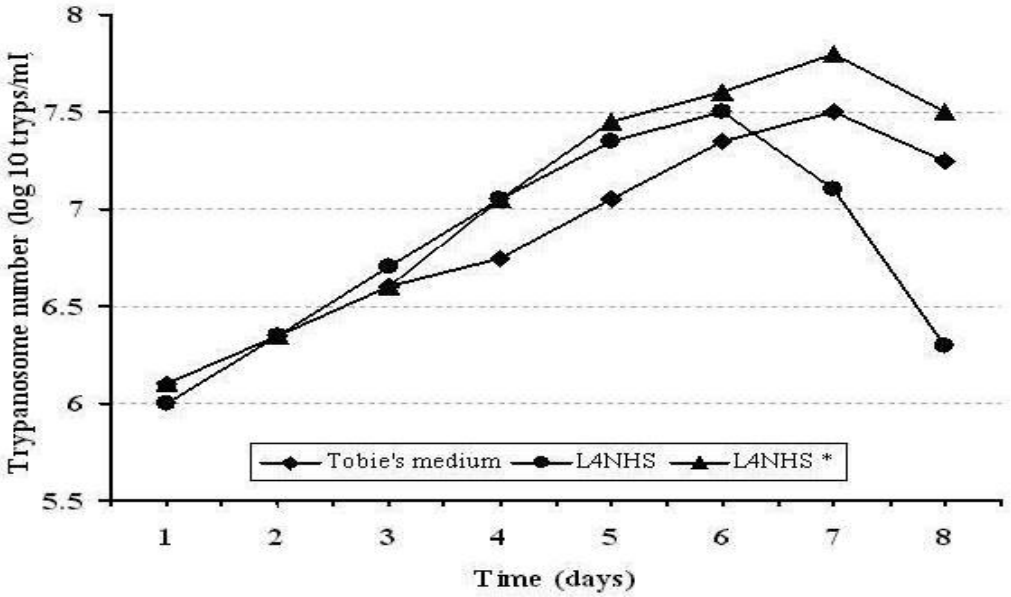

Figure 3: Development in growth of *T. danilewskyi* strain FCc-1 in various culture media. The strain FCc-1 was cultivated in Tobie's and L4NHS media separately. The same strain when was adapted to culture conditions in Tobie's medium, was cultivated in L4NHS medium marked as L4NHS*. n=16.

Table 1. Chemical composition of Tobie's medium used for the initiation of culture and then cultivation of *T. danilewskyi* strain FCc-1 culture

Bacto tryptose (Difco)	3.0 g
Na Cl	0.8 g
$Na_3 PO_4.12 H_2O$	1.0 g
Bidistilled water	200 ml
Dissolve the ingredient and adjust pH 7.6 and add Bacto agar (Difco)	3.0 g

Table 2. Chemical composition of monophasic liquid culture medium (L_4NHS) used for the rapid cultivation of *T. danilewskyi* strain FCc-1

Proteose peptone (Difco)	3.0 g
Liver heart digest (Oxide)	0.5 g
Yeast extract (Difco)	1.0 g
Na Cl	1.0 g
Bidistilled water	200 ml
Dissolve the ingredient and then add	
Rabbit serum (heat inactivated)	10 ml
Erythrocyte lysate (10 % in water)	20 ml
MEM Vit. Mixture (Gibco)	10 ml

Adjust pH to 7.5

Table 3: In vitro culture initiation and cultivation of *Trypanosoma danilewsky*i strain FCc-1 in different culture media at 20 ºC. Live trypanosomes present or absent (+/ or -/) and dividing forms present or absent (/+ or /-).

Culture media	Time (days)							
Culture initiation	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Tobie's medium	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+
Cultivation								
Tobie's medium	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+
L4 NHS	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+

DISCUSSION

The blood stream forms of fish trypanosomes undergo multiplicative divisions most commonly by binary fission, dividing sometimes into equal trypomastigotes and mostly into unequal, epimastigotes and trypomastigotes in the culture media (Brün *et al*., 1981, Cunningham *et al*., 1981, Davie *et al*., 1992, Jones and Woo, 1991, Ahmed *et al*., 2001). During culture initiation of *T. danilewskyi* strain FCc-1 in Tobie's culture medium, trypanosome propagation was significant because of Locke-Krebs overlay containing many organic and inorganic salts and MEM Vitamin Solution (Gibco, Invitrogen Corporation, USA).

Trypanosome multiplication and growth was significantly high ($P<0.05$) in monophasic media, when only adapted trypanosomes were inoculated, either in blood derived (L4 NHS) or synthetic (RPMI 1640 & GLSH-DCA). Similar results were obtained by Cunningham and Honigberg (1977) and Cunningham *et al.* (1981) in monophasic media's where trypanosome yield was always higher than biphasic media. The yield of *T. danilewskyi* strain FCc-1 in Tobie's medium (1 ml overlay) was 4 x 106 trypanosomes/tube (weekly) while a similar inoculum produced 1.2 x 107 flagellates/tube (2 ml) in L4 NHS medium within the same time. The yield of trypanosome from L4 NHS was slightly higher than from Tobie's medium but shifting adapted trypanosome from Tobie's medium to L4 NHS, resulted in a rapid growth and a yield of 1.2 x 107 trypanosomes/tube/week was obtained in L4 NHS. This medium was found satisfactory because of its inexpensive ingredients, continuous growth, and easy preparation. It is a liquid monophasic medium; the yield was more than 10 million trypanosomes / tube (2 ml) without any change of culture medium problem. These findings are corroborating with Zweygarth *et al*. (1989) where they obtained excellent growth during the culture of *Trypanosoma brucei brucei* and *T. brucei evansi*. While other culture media like GLSH-DCA and RPMI 1640, both contain expensive ingredients and produced less live trypanosomes in similar amount of culture media (Ahmed *et al*., 2001)

It is well known that the end products of metabolism in protozoan consist of carbon dioxide, pyruvate, urea and ammonia (Bryant, 1982; Larsen *et al*., 1988). These metabolites are toxic to parasites when they accumulate in the media and are responsible for the lyses of flagellates and formation of round forms (Li & Woo, 1991) in the old cultures. The percentage of lysed cells and round forms may increase due to the facts that (i) the cultures become nutritionally depleted and (ii) toxic metabolites accumulate in the culture media. These round forms are mostly without nucleus or kinetoplast and are devoid of dividing capabilities. Similar round forms were also seen in 12 days old culture of *T. danilewskyi* strain FCc-1 both in Tobies' and L4NHS culture media.

Conclusion: The present study revealed that *T. danilewskyi* strain FCc-1 can easily be cultured in monophasic liquid medium (L4NHS) for large scale production of flagellates meant for any future biochemical or molecular studies. It was also considered best among other tested media during this study and other previous studies because it contained in-expensive ingredients that can be made easily available locally.

Acknowledgements: The first author (*M. S. Ahmed*) obtained an IRO grant from the Katholieke Universiteit Leuven, Belgium. Authors are obliged by using the Tissue Culture Facility at Laboratory of Protozoology, Institute of Tropical Medicine and Hygiene, Antwerp, Belgium.

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