A SIMPLE TECHNIQUE FOR THE *IN VITRO* CULTIVATION OF NOCTURNALLY SUBPERIODIC *BRUGIA MALAYI* INFECTIVE LARVAE

Pongsri Tippawangkosol, Wej Choochote, Doungrat Riyong,
Atchariya Jitpakdi and Benjawan Pitasawat

Department of Parasitology, Faculty of Medicine, Chiang Mai University,
Chiang Mai, Thailand

Abstract. A simple system for the *in vitro* cultivation of nocturnally subperiodic *Brugia malayi* was developed. The manner of cultivation consisted of a 1:1 (v/v) mixture of Iscove's Modified Dulbecco's medium and NCTC-135 medium supplemented with 20% fetal bovine serum by using candle jar incubation at 37 ^{o}C instead of CO_2 incubator. Changing the media: every 2 days, 3 days and changing media on day 7, then every 2 days produced a larval survival rate of 50% (70/140) on day 10, 49% (82/166) on day 6, and 53% (105/200) on day 9. With this technique, up to 50% of the infective stage larvae (L3) survived for up to 10 days and had long life for at least 27 days in all experiments with low larval survival rate in the fourth week. In addition, the culture system promoted molting L3 to fourth stage larvae (L4) after 7 days, as shown by light microscope.

INTRODUCTION

Lymphatic filariasis caused by *Wuchereria bancrofti* and *Brugia malayi* is a major public health problem in the tropics (WHO, 1992). Approximately 120 million of the world's population are infected with filarial parasites and 1,100 million people are at risk of infection (Addiss, 1998).

A complex culture supplement of serum and cells, which promotes the molting of the infective stage larvae (L3) to fourth stage larvae (L4) of the human filarial parasite *B. malayi in vitro*, has been developed during the past few years (Mak *et al*, 1983; Riberu *et al*, 1990; Smillie *et al*, 1994; Falcone *et al*, 1995; Smith *et al*, 2000; Smith and Rajan, 2000). The most promising cultures were those that used various mammalian cell lines as feeder layers and human serum that allowed the growth and molting of L3 to the late L4 larval stage. In recent years, the study of a serum-free system for the *in vitro* cultivation of *B. malayi* infective stage larvae has been pursued (Smith *et al*, 2000). Although that culture system promoted molting L3 to the L4 larval stage, and the larvae survived for upward of 2 weeks, it needed a culture additive that provided a rich source of nutrients for larval development, including a basidiomycetous yeast and fatty acids in an RPMI-1640 medium. We report on a simple serum-free *in vitro* cultivation system that is effective in the absence of a yeast supplement.

Correspondence: Pongsri Tippawangkosol, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail : ptippawa@mail.med.cmu.ac.th

MATERIALS AND METHODS

Nocturnally subperiodic (NSP) *B. malayi* L3 (Narathiwat strain) were obtained from laboratory-reared *Aedes togoi* that had been fed on microfilaremic cats 12 days earlier. The mass colonization of *Ae. togoi* and the rearing of mosquitos followed the techniques described by Choochote (1981). The whole bodies of mosquitos that had been infected for 12 days were dissected using an aseptic technique (Suwan *et al*, 1993). Mass dissection was carried out in a Hank Balanced Salt Solution (HBSS) with antimicrobial agents (10,000 units/ml of penicillin G; 10,000µg/ml of streptomycin disulfate; 25µg/ml of amphoteracin B) in a sterile Petri dish (30mm diameter). *In vitro* cultivation was performed according to the method of Smillie *et al* (1994) with modification and/or simplification. The culture media used to support the growth and development of NSP *B. malayi* L3 to L4 or adults consisted of a 1:1 (v/v) mixture of Iscove's Modified Dulbecco's medium (IMDM, Sigma) and NCTC-135 medium (Sigma) supplemented with 20% heat-inactivated fetal bovine serum (NI-FBS), a mixture of antimicrobial agents, and 25mM HEPES (Sigma). We modified the culture system by using candle jar incubation at 37^{o}C instead of CO_2 incubator. With this method, *in vitro* cultivation could be performed in laboratories that lack a CO_2 incubator. *B. malayi* L3 were washed by repeated transferring them to fresh media with a mixture of antibiotic agents in a culture Petri dish. Twenty L3 larvae were cultured in a Petri dish containing 2 ml of culture media (NI-FBS) containing a mixture of antimicrobial agents. Plates were placed in a candle jar (a glass desicator equipped with a stopcock and a white candle). The white candle was lit, the cover was put on, and the

stopcock was opened. When the candle went out, the stopcock was closed immediately. By this method, an atmosphere of low oxygen was produced. The candle jar was then incubated at 37°C in an incubator. Larvae were counted when the culture media were changed: every 2 days (experiment 1), every 3 days (experiment 2) and changing media on day 7, then changing media every 2 days (experiment 3). Morphological changes or molting were observed by light microscopy. For light microscopy, the larvae were transferred to fresh culture media, fixed in hot 70% ethanol, cleared first in 5% glycerin in 70% ethanol and then in 20% glycerin in 70% ethanol, and mounted in glycerin on a slide. Measurements and morphological studies were carried out using light microscopy, camera lucida, and a calibrated ocular micrometer.

RESULTS

The result of using our simplified *in vitro* cultivation system and changing media; every 2 days (experiment 1), 3 days (experiment 2) and changing media on day 7, then changing media every 2 days (experiment 3) produced larval survival rates of 50% (70/140) on day 10, 49% (82/166) on day 6, and 53% (105/200) on day 9 respectively (Table 1). Changing the media by the 3 different methods did not unduly influence the survival rate of the larvae. With this simple *in vitro* technique, some 50% of L3 survived for up to 10 days and had long life for at least 27 days in all experiments, although the survival rate of the larvae fell in the fourth week. A comparison of the survival rate of NSP *B. malayi* larvae and their morphometric measurements is given in Tables 2 and 3 and in Figs 1 and 2. The morphometric measurements (of 20 larvae) that were made on each experiment of different days after *in vitro* cultivation indicated that the larvae cultivated in this simple technique for 7 days were significantly longer and had wider bodies, than the L3 larvae. The means body length and body width of the *B. malayi* larvae cultured in the simple media (NI-FBS) used in this study after 7 days were 2,040 and 32.3μm, respectively, which increased from L3 in body length and width of 26.6% and 39.1%, consecutively. In contrast, the mean ± SD of the anal ratio (L/W) of the larvae cultured after 4, 5, 6, and 7 days in NI-FBS was significantly lower than those of L3 (2.53 ± 0.4, 2.61 ± 0.4, 2.67 ± 0.4, and 2.63 ± 0.4 *vs* 3.43 ± 0.4 respectively).

In the simple *in vitro* culture system, the first molting and L4 generated in culture were seen on day 7 (Fig 3) by light microscopy. Morphological changes during the first week of culture included a characteristic change in the shape, from the tapering head of L3 to the globular head of the fourth stage after molting (Figs 3A and C). In addition, the characteristic terminal and two sublateral papillae of the L3 were lost at the molt and the tail end of the fourth stage appeared rounded (Figs B and D), as described by Mak *et al* (1983) and Zaman (1987).

DISCUSSION

The simple *in vitro* coculture system without cells or any culture additives that serve as a rich source of nutrients for larval development was developed by using the basic culture medium (NI-FBS). Furthermore, cultivation of these parasites in a candle jar is more convenient in laboratories that lack CO_2 incubators. Our results showed that the molting of L3 to L4 occurred after 7 days in our simple medium. A recent study reported that a serum-free *in vitro* system, consisting of RPMI-1640 supplemented with fatty acid and a basidiomycetous yeast, promoted molting after 7 days (Smith *et al*, 2000). Several studies have shown that the *in vitro* cultivation of *B. malayi* larvae using different media or various additives may produce different rates of parasite growth and development (Mak *et al*, 1983; Riberu *et al*, 1990; Smillie *et al*, 1994; Smith *et al*, 2000; Smith and Rajan, 2000). Molting of L3 to L4 occurred after 7 days in NI supplemented with 10% FBS, 10% pooled human serum from hospital patients and 10% human serum from a single individual, whereas NI supplemented with bovine albumin fraction-V and 10% commercially obtained human serum did not support molting (Smillie *et al*, 1994). Falcone *et al* (1995) reported that the first molting of *B. malayi* larvae was seen after 8 days when cultured in a cell-free system, and at 8-10 days when cocultured with human dermal fibroblasts and human T-cell line Jarkat; this molting time may be as much as 14 days when coculture with only human dermal fibroblasts is tried. A similarly long molting time was seen when *B. malayi* larvae were cultured in RPMI-1640 supplemented with 10% human AB serum and an LLC-MK_2 Rhesus monkey kidney continuous cell line feeder layer allowed the growth and molting of L3 to L4 at 14 days and kept the larvae alive for 54 days (Mak *et al,* 1983). Even though the simple *in vitro* technique in this study supported the molting of L3 to L4 in the first week, the survival time of the larvae was considerably less than that of larvae cultured in media that have various supplements, as shown by previous studies (Mak *et al*, 1983; Riberu *et al*, 1990; Smillie *et al*, 1994; Falcone *et al*, 1995; Smith *et al*, 2000). The relatively low larval survival rate in the fourth week of our study could have been due to the results of lacking the important nutrient to promote the molting or to stimulate specific host factors. In

Table 1
Comparion of the survival rates of NSP *B. malayi* larvae cultured in a 1:1 mixture of NCTC-135 and Iscove's Modified Dulbecco's Medium supplemented with 20% FBS in three different experiments.

Days of culture	Larval survival (% survival rate)		
	Experiment 1[a]	Experiment 2[b]	Experiment 3[c]
Day 0	140 (100)	166 (100)	200 (100)
Day 1			
Day 2	107 (73)		
Day 3		95 (57)	
Day 4	102 (73)		
Day 5			
Day 6	88 (63)	82 (49)	
Day 7			132 (66), 20 (67)[d]
Day 8	79 (56)		
Day 9		68 (41)	105 (53)
Day 10	70 (50)		
Day 11			90 (45)
Day 12	66 (44)	53 (32)	
Day 13			89 (45)
Day 14	60 (43)		
Day 15		39 (23)	82 (41)
Day 16	47 (34)		
Day 17			70 (35)
Day 18	46 (33)	23 (14)	
Day 19			40 (20)
Day 20	41 (29)		
Day 21		18 (11)	32 (16)
Day 22	25 (18)		
Day 23			13 (7)
Day 24	16 (11)	14 (8)	
Day 25			6 (3)
Day 26	12 (9)		
Day 27		2 (1)	1 (1)
Day 28	7 (5)		
Day 29			-
Day 30	3 (2)	-	
Day 31			
Day 32	-		

[a] Changing media every 2 days;[b] changing media every 3 days;[c] changing media on day 7, then changing media every 2 days;
[d] Repeated experiment

addition, the L4 did not develop further. The development of the L4 to the young adult stage was seen in Falcone's system after 37 days (Falcon *et al*, 1995). Furthermore, L4 was found to be molting to sexual maturity after 60 days in Riberu's system (Riberu *et al*, 1990). However, the increases in length and width of *B. malayi* larvae after 7 days in NI-FBS were greater than those of the parasites cultured in RPMI-1640 supplemented with 10% human AB serum and an LLC-MK_2 Rhesus monkey kidney cell line feeder layer (Mak *et al*, 1983).

A simple *in vitro* culture technique for NSP *B. malayi* was developed. The system is inexpensive, straightforward, and does not require a CO_2 incubator. In addition, it may be of benefit to those studying the *in vitro* efficacy of filaricidal drugs and their sites of action. Moreover, the technique may produce stage-

Table 2
Comparative morphometric measurements (μm) of NSP *B. malayi* larvae cultured from infective larvae (L3) in a 1:1 mixture of NCTC-135 and Iscove's Modified Dulbecco's medium supplemented with 20% FBS for 7 days (number of larvae = 20).

Days of culture	Length (μm)[a]			Width (μm)[a]		Anal ratio
	Body	EJ	Anal	EJ	Anal	L/W
Day 0	1,612±108 (1,430-1,760)	594±55 (550-660)	54±4.9 (46-65)	23±1.5 (22-27)	16±1.2 (14-19)	3.43±0.4 (2.8-4.4)
Day 4	1,671±80[b] (1,430-1,760)	599±56 (550-660)	51±8.0 (32-65)	27±2.1[b] (24-32)	19±1.8[b,c] (16-22)	2.63±0.4[b] (2.0-3.4)
Day 5	1,738±184[b,c] (1,210-1,980)	654±90[b] (440-880)	55±6.6 (41-65)	27±2.8[b] (19-30)	21±1.9[b,c] (19-24)	2.67±0.4[b] (1.7-3.3)
Day 6	1,930±153[b,c] (1,650-2,090)	704±80[b] (660-880)	58±7.3[b,c] (38-70)	30±2.4[b,c] (27-35)	23±1.7[b,c] (19-24)	2.61±0.4[b] (1.6-3.3)
Day 7	2,040±112[b,c] (1,870-2,200)	671±97[b] (550-880)	64±11.1[b,c] (41-95)	32±7.0[b,d] (22-51)	26±2.6[b,c] (22-32)	2.53±0.4[b] (1.5-3.1)

EJ = Esophago-intestinal junction
[a] Mean ± SD / range ; [b] Significantly different from L3 ($p< 0.05$);
[c] Significantly different from all larvae above ($p< 0.05$); [d] Significantly different from all larvae above ($p< 0.05$), except Day 6 larvae.

Table 3
Mean morphometric measurements (μm) ± SD of NSP *B. malayi* larvae cultured from infective larvae (L3) in a 1:1 mixture of NCTC-135 and Iscove's Modified Dulbecco's medium supplemented with 20% FBS for 7 days (number of larvae = 20).

Days of culture	Body length	Body width	Percentage increase from L3	
			Length	Width
Day 0	1,621±108 (1,629±68)[a]	23.4±1.5 (28.3±0.4)[a]	-	-
Day 4	1,671±80[b,c]	27.3±2.1[b]	3.7	17.4
Day 5	1,738±184[b,c]	26.9±2.8[b]	7.8	17.4
Day 6	1,930±153[b,c]	30.0±2.4[a,c]	19.7	30.4
Day 7	2,040±24[b,c] (1,902±24)[a]	32.3±7.0[a,d] (36.1±0.9)[a]	26.6 (16.8)[a]	39.1 (27.5)[a]

[a] Mean measurements (μm) ± SD of NSP *B. malayi* larvae cultured from infective larvae (L3) in RPMI-1640 with 10% human AB serum and LLC-MK_2 Rhesus monkey kidney continuous cell line feeder layer, significantly different from L3 (*t* test, $p<0.05$) (Mak *et al*, 1983)
[b] Significantly different from L3 ($p<0.05$)
[c] Significantly different from all larvae above ($p<0.05$)
[d] Significantly different from all larvae above ($p<0.05$), except Day 6 larvae

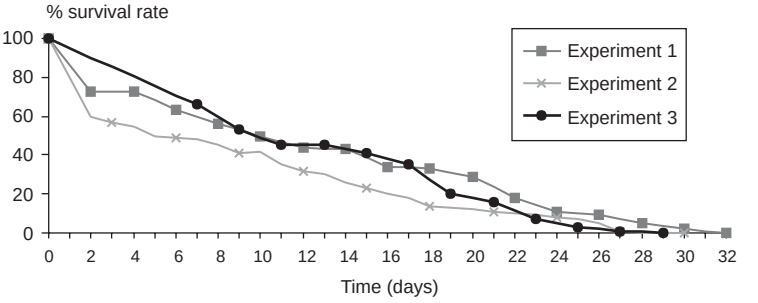

Fig 1- Survival rate of NSP *B. malayi* cultured in three different experiments.

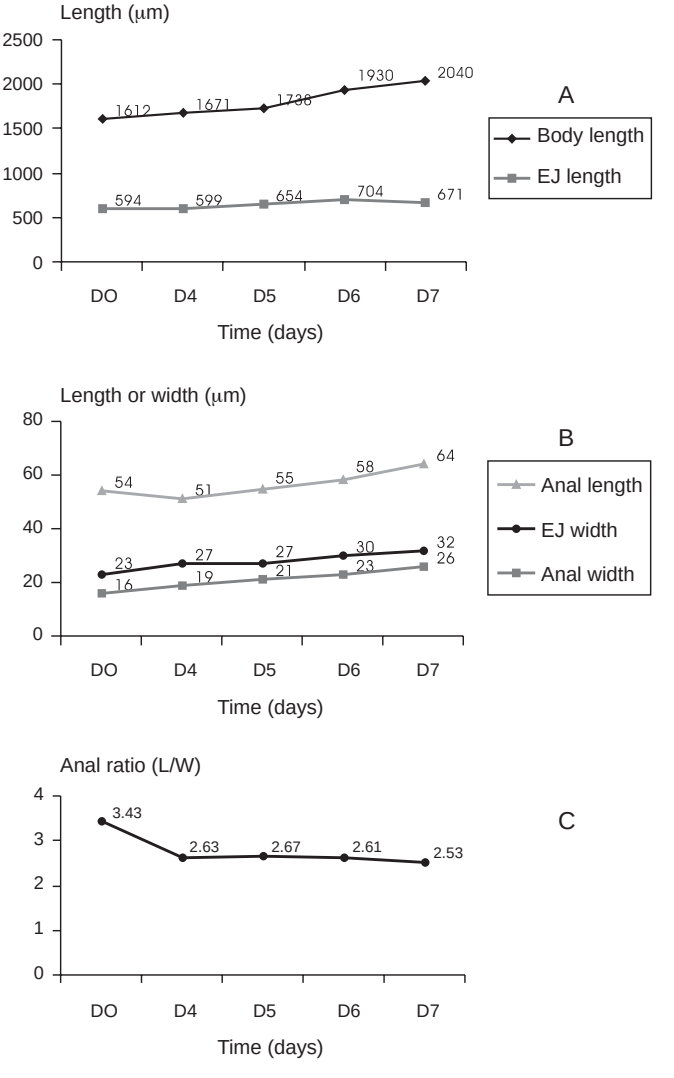

Fig 2- Development of NSP *B. malayi* larvae cultured from infective larvae (L3) in a 1:1 mixture of NCTC-135 and Iscove's Modified Dulbecco's medium supplemented with 20% FBS *in vitro* for 7 days. (A) Body length and esophago-intestinal junction (EJ); (B) Anal length, EJ width, and anal width; (C) Anal ratio (L/W).

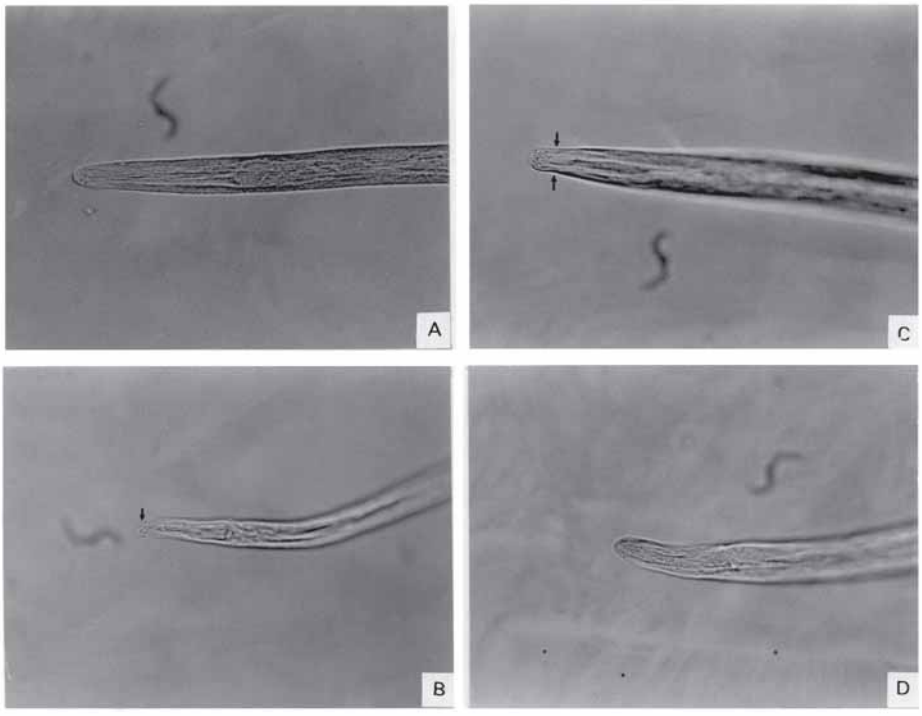

Fig 3- NSP *B. malayi* larvae; (A) Tapering head and anterior end of infective larva (L3) recovered from *Ae. togoi*, 12 days post-infection; (B) Posterior end of L3, showing three terminal papillae (arrow); (C) Globular head (arrow) and anterior of a fourth stage larva (L4) recovered after 7 days *in vitro* culture of L3 in a mixture of 1:1 (v/v) Iscove's Modified Dulbecco's medium and NCTC-135 medium supplemented with 20% fetal bovine serum; (D) Posterior end of L4 showing rounded posterior end and the absence of terminal papillae.

specific antigens, including molting fluid and excretory and secretory antigens, which might prove useful in immunological studies.

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