

IN VITRO CULTIVATION OF THIRD STAGE LARVAE OF *WUCHERERIA BANCROFTI* TO FOURTH STAGE: INFLUENCE OF SOME PHYSICO-CHEMICAL FACTORS

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Abstract. It has been reported that third stage larvae (L3) of *Wuchereria bancrofti* strain from Jakarta, molted to the fourth stage (L4) *in vitro*, in a simple culture medium supplemented with 10% human serum. In the present study, this culture medium has been used to examine the effects of some physico-chemical parameters on larval growth, development and molting of *Wuchereria bancrofti* from India. Lymph at 10% concentration enhanced the *in vitro* survival time of larvae. Molting of larvae from L3 to L4 stage has been obtained using human fetal lung cells in cellular co-culture and as a source of conditioned medium. Given these improvements in the medium supplementation, it has been observed that the age of L3s (duration of L3s maintenance within the mosquitos) is one of the most important parameters for the development of L3s *in vitro*. No molting was observed when one day L3s were used whereas, molting occurred with one or two weeks old L3s. On the contrary, when more than 3 weeks old L3s were used molting failed to occur even though duration of survival of L3s was improved and in this case, most of the larvae were degenerated.

INTRODUCTION

Cultivation of filarial parasites is useful for drug testing, and for immunological and metabolic studies. Filarial parasites cultured *in vitro* have been used as a source of secretory, excretory and molting antigens of worms, free of immunological factors from the host. Some species, *Brugia pahangi* and *Dipetalonema viteae* have been cultivated successfully from third to fifth stage (Franke and Weinstein, 1983; Mak *et al.*, 1983). A recent report of *in vitro* cultivation of *B. malayi* to sexually mature adult stages has been a significant development (Riberu *et al.*, 1990). With *Wuchereria bancrofti*, the commonest filarial parasite causing lymphatic filariasis in most areas of the tropics, only molting and limited growth has been achieved with a strain from Jakarta, Indonesia (Franke *et al.*, 1987, 1990). No attempts have been made for the *in vitro* cultivation of other geographic strains of *W. bancrofti*, especially from India. Hence, We studied the *in vitro* cultivation of *W. bancrofti* L3 stage larvae from Pondicherry (South India) under different *in vitro* culture conditions. The survival and development of larvae from different cultures were compared.

MATERIALS AND METHODS

Preparation of inoculum

Wuchereria bancrofti strain from Pondicherry was used in all the studies. The mosquito vector utilized was laboratory reared *Culex quinquefasciatus*. The mosquitos were fed artificially on microfilaremic blood collected from patients (Wade, 1976). The fed mosquitos were held in cages at $27 \pm 2^\circ\text{C}$ for 14 days, when all the parasites were at third stage (L3). The infected mosquitos were then anesthetized lightly with ether, dissected and held in RPMI 1640 medium (Sigma, St Louis, USA) containing antibiotics solution (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 0.25 $\mu\text{g}/\text{ml}$ fungizone: Sigma, St Louis, USA). Once L3s were released into the medium (about half an hour), they were separated under a microscope and washed 3 times with the above medium containing antibiotics. They were again washed 3 times under sterile conditions with basal culture medium (1:1 (v/v) mixture of NCTC 135 and Iscove's modified Dulbecco's medium (Sigma). After the final wash, 50 larvae each were inoculated into culture flasks containing 5 ml of complete culture medium.

Culture media

The complete culture medium consisted of 1:1 (v/v) mixture of NCTC 135 and IMDM containing 2mM glutamine, 15mM glutathione and 1 × antibiotics solution. This medium was supplemented with 10 or 20% foetal bovine serum (Tecno-nara, Northumbria Biologicals, UK) or human serum from Indian donors. In some cases the medium was supplemented with 10 or 20% lymph taken from infected donors. In order to see the influence of cellular co-culture on larval development, human foetal lung cells (HFL) or Rhesus monkey kidney cells (LLCMK2) (provided by the National Facility for Animal Cell and Tissue Culture, Pune, India) was used as feeder layer in some culture. The same cell lines were also used as a source of conditioned medium.

Maintenance of cultures

The culture flasks used were 25 cm² T flasks (Nuncleon, Intermed, Denmark). Cultures were kept stationary at 37°C in an atmosphere of 5% CO₂ and 95% air in an incubator. The culture medium was changed every 3 days by removing half of the old medium and replacing with same quantity of fresh medium. The motility (sluggish or active) and any changes in morphology of the larvae were examined daily with a phase-contrast inverted microscope. When cultures were terminated the larvae were fixed in hot 70% alcohol, transferred to a mixture of 5% glycerine in 70% alcohol and then to a mixture of 20% glycerine in 70% alcohol. The alcohol was allowed to evaporate and the larvae were mounted in glycerine and examined for the state of development and measured for length and width (Lim and Sim, 1983).

RESULTS AND DISCUSSION

Influence of different sera on the survival of larvae

In order to select an appropriate serum for the cultivation of third stage larvae of *W. bancrofti*, different sera viz, fetal bovine serum and sera from different human individuals (microfilaria positive and normal), with and without inactivation, at different concentrations, were tested, using the *in vitro* culture system proposed by Franke *et al* (1987). One sample of human sera, viz normal human serum gave prolonged duration of survival (5 days) compared to other samples indi-

vidually and pooled together (3 days). Serum has been shown to be an essential supplement for survival and molting of L3s. However, considerable variations among different lots of serum in their ability to support L3 growth and development has been reported (Franke *et al*, 1990). In our study, the duration of survival of larvae was far less than that reported for the strain of *W. bancrofti* from Jakarta, although cultured in the same medium, by Franke *et al* (1990). This indicates that different geographic strains differ in their amenability to a particular culture system. Hence, we attempted to improve the culture system, further.

Influence of the age of L3s

Microfilariae develop to third stage in the mosquito host 12 to 14 days after their ingestion and usually the L3s were taken immediately after their development (1 day old) for *in vitro* cultivation studies by other investigators (Franke *et al*, 1987; Riberu *et al*, 1980; Baird *et al*, 1991). In our recent studies it was noted that L3s of *W. bancrofti* were alive and active up to 36 days after maturation into L3 *ie* as long as the host mosquitos, *Cx. quinquefasciatus*, survived (unpublished data). We tested 1, 7, 14 and 21 days old L3s, in order to see the effect of age of L3s and hence the prolonged contact of L3s with the internal environment of mosquito host, on subsequent *in vitro* development. The results are presented in Table 1.

We observed an increase in the length of the larvae when 7, 14 and 21 days old L3s were used for inoculation (1.9mm). However, when 1 day old larvae were used there was no marked increase in their length (1.5 mm). Also, there was a substantial improvement in the duration of survival of larvae when older ones were used for inoculation. The third stage larvae inoculated when they were 1 day old survived for 7 days whereas when they were inoculated at 7, 14 and 21 days old they survived for 20 to 35 days.

When 7 and 14 days old L3s were used for culturing we observed molting in 2% of L3s after 3 and 10 days of inoculation, respectively, unlike in the case of 1 or 21 days old larvae in which no molting was observed. Very little information is available in the literature concerning the process and time of formation of the L4 cuticle. In the case of *Dirofilaria immitis*, certain culture conditions were necessary for synthesizing the fourth stage epicuticle and also for the shedding of the

Table 1

Influence of age* of *Wuchereria bancrofti* L3s on their development *in vitro*.

Age of L3s (days)	Length (mm)	Remarks
1	1.5	One week survival-no changes
7 and 14	1.85 - 2.1	Three weeks survival 2% of L3s molted to L4s
21	1.8 - 2.0	Long term survival (35 days) and degeneration of larvae

* L3s are maintained for 1, 7, 14 and 21 days in the mosquito host before inoculation in the culture medium.

third stage cuticle (Abraham *et al*, 1990). In the present study, as one to two weeks old larvae molted to L4 stage, it appeared that some modifications occurred in L3s due to their prolonged stay within mosquitos, helping the transition and adaptation of L3s to the *in vitro* culture conditions. The younger larvae did not molt probably due to the absence of such modification.

All the larvae inoculated at the age of 21 days were alive and active for one week. Thereafter, 20% of the L3s showed vacuolation and signs of degeneration (Plates 1a-1b). Even after the onset of the degeneration, L3s remained active for at least 7 days. Lok *et al* (1984) observed that if the larvae of *D. immitis* failed to molt *in vitro* at the appropriate time, they became first vacuolated and then inactive. It appears that some factor(s) which induce the molting may be missing and the viability is lost in the older L3s (3 weeks old). However, in this case, live L3s were seen till 35 days after inoculation. thus a long term survival of L3s has been achieved.

Influence of human lymph

Filariae have highly specific sites of development within mammalian hosts and successful *in vitro* culture may require the simulation of the micro-environment (Cupp, 1991). *W. bancrofti* larvae develop to adult stage in the lymphatic system and hence we used human lymph at 10 and 20% level, as a supplement. Since the 21 days old L3s survived *in vitro* for longer duration, even though did not molt, we used larvae of this age to see if the lymph could induce the molting process. In both 10 and 20% lymph containing media the survival of larvae was for 43 days, while in medium without lymph it was for 37 days. Thus,

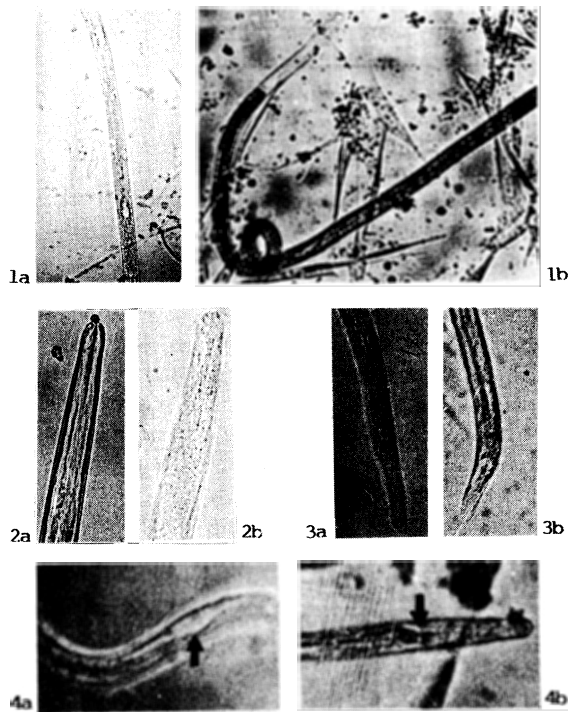


Fig 1—21 days old L3 of *W. bancrofti* showing vacuolation (1a) and degeneration (1b) grown in *in vitro* culture condition. × 10.

Fig 2—Comparison of tapering anterior end of L3 (2a) and globular head of early stage L4 (2b) of *W. bancrofti* grown in *in vitro* culture conditions. × 20.

Fig 3—Comparison of caudal end of male (3a) and female (3b) L4s of *W. bancrofti* after 20 days of *in vitro* culture. × 20.

Fig 4—Portion of male *W. bancrofti* L4 showing testis (4a) and portion of female L4 showing the vulva (4b) after 20 days of *in vitro* culture. × 40.

there was a little improvement in the duration of survival of L3s in culture when lymph was supplemented. In the culture without lymph 25% of L3s were found to be alive after 34 days of culture. But when lymph was added 50% of L3s were alive after the same period of cultivation. Still, many L3s were found to be degenerated in both the cases after 15 days in culture. Although supplementing the lymph to the culture medium did not induce molting, it helped to increase the duration and percentage survival of the L3s. The protein content of the lymphatic fluid is lower than that of serum (Harper *et al*, 1979) but it appears to contain different components which may be vital to the survival of L3s. Hence, supplementing the lymph to the culture medium appears to be advantageous for *in vitro* cultivation of L3s.

Influence of cellular co-culture

Since we observed molting of 2% of the larvae when 7 to 14 days old L3s were used for inoculation, we repeated the same experiments but with cellular co-culture. In fact, medium conditioned with cells would provide metabolites or growth factors different from those provided by serum and which may be vital for larval development. It has also been reported by Cupp (1991) that cellular co-culture is favorable for larval development, especially because of its ability to reduce oxygen concentration of the medium. LLCMK2 cell line has been used successfully for the development of fourth stage larvae of *B. malayi* and *B. pahangi* (Mak *et al*, 1983). Cross *et al* (1981) reported the maximum recovery of

worms from the lungs of gerbils inoculated with *W. bancrofti* L3s. Hence we used lung cells (human fetal lung cells) as feeder layer. Along with a standard culture containing no cells, L3s were inoculated to a set of flasks with cell line (HFL and LLCMK2) and another with cell line supplemented with 40% of conditioned medium from the respective cells. Percentage of larvae surviving after 7 days and average length of larvae at the end of the culture are presented in Table 2.

Most of the larvae (94 to 99%) were alive and highly active for one week in all the cultures. Twelve days after inoculation some larvae (4%) were found to be degenerated in both the cultures, with and without HFL cells. In both cases many larvae had become thicker, granulated and sluggish in movement. By day 16, in all the cultures the sluggish larvae became very active again. On day 20, 30% of the larvae were found to be longer than the others, *ie* 2.1 mm, in cultures containing HFL cells enriched with 40% conditioned medium.

Microscopic examination of the larvae showed that the shape of head of many larvae was round unlike the normal tapering head of L3s (Plates 2a, 2b), the stoma more pronounced and the oesophagus granular. Also, primordia of testis, the curved tail in males and appearance of vulva in females were seen. These developments indicated that these larvae were of L4 stage (Plates 3a, 3b, 4a, 4b). When the L3s were grown with LLCMK2 cells, only 60% were alive on day 7. In this case also some larvae became active again after a sluggish phase of a week. On day 20, 3 or 4 larvae were found to be longer (1.85mm), thin and with-

Table 2

Effect of cell line, HFL and LLCMK2, used as feeder layer and as a source of conditioned medium, on the survival and development of *Wuchereria bancrofti* L3s *in vitro*.

Culture system	% of L3 survival after 7 days	average length (mm)
Complete medium (CM)	94%	1.6
(CM) + HFL cells	97%	1.77
(CM) + HFL cells + cond m*	99%	1.87 (max.2.1)
(CM) + LLCMK2 cells	61%	1.68
(CM) + LLCMK2 cells + cond m	90%	1.69

*cond m : conditioned medium with HFL or LLCMK2 cell line.

out granulation in both LLCMK2 cells containing flasks (with and without conditioned medium).

Our results show that HFL cell line seems to be more suitable for the molting of L3s than LLCMK2 cell line. Further, supplementation of conditioned medium was more favorable. This indicates that apart from reducing the oxygen concentration in the culture system (Cupp, 1991) the cells also provide certain chemical factors necessary for the development of the larvae. We have also observed that the dead cells sticking to the larvae helped them to cast their cuticle.

It has been reported that in jirds L3 of *W. bancrofti* molt between 8 and 11 days post-inoculation (Ash and Schacher, 1971), and in *in vitro* conditions between 10 and 20 days of culture (Franke *et al*, 1987). We observed a non-synchronous molting of L3s, between 3 and 20 days of inoculation. The average length of L4 soon after molting was 2.1 mm, similar to that observed by Ash and Schacher (1971) for early fourth stage larvae of *W. bancrofti* grown *in vivo*.

Franks *et al* (1987, 1990) have reported the growth and development of *W. bancrofti* L3s to L4 stage under *in vitro* conditions using a simple culture system containing human serum. We attempted the culturing of Pondicherry strain of *W. bancrofti* by adopting the same culture method but failed to get the same level of development. Even different human sera available, when supplemented, did not give satisfactory results. Hence we tried to improve the culture conditions. Higher concentrations of serum and other supplements such as lymph appeared to improve the survival and development of larvae. In contrast to what has been taken into account by other investigators, we studied the effect of age of L3s in mosquitoes on *in vitro* cultivation. According to the results obtained, it appears that the age of L3s is one of the important parameters for development of L3s *in vitro*. The choice of the appropriate human serum and cell line (HFL cells) as feeder layer was also important to provide specific growth factors and physical conditions that improved development and molting of *W. bancrofti* L3s. The culture system developed in this investigation will be useful for the *in vitro* screening of antifilarial compounds and for obtaining excretory-secretory antigens.

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