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# The development of *Dirofilaria immitis* in cultured malpighian tubules

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## Summary

A technique is described for the cultivation in vitro of *Dirofilaria immitis* to the second larval stage within malpighian tubules excised from mosquitoes 20–24 h post infection. The malpighian tubules were removed from mosquitoes under aseptic conditions, washed and inoculated into a variety of tissue culture media, both insect and mammalian. In some cultures a feeder layer of *Aedes malayensis* cells was included. The 24 h larvae developed to the second stage only in Schneider's Drosophila medium containing 20% foetal bovine serum, irrespective of whether a cell layer was included in the cultures. Development of the larvae to the infective third stage was not observed.

*Key words: Dirofilaria immitis;* in vitro cultivation; excised malpighian tubules; Schneiders's Drosophila medium; second stage larvae.

The microfilariae of a variety of species of filarial nematode, isolated from infected blood or from skin snips, may be stimulated to develop in vitro to the late first larval stage (or the 'sausage' stage) but there are few reports of development occurring in vitro beyond this stage (see reviews by Taylor and Baker, 1968; Hansen and Hansen, 1978; Pudney and Varma, 1980). Wood and Suitor (1966) reported the development of the microfilariae of *Macacanema formosana* to the second and possibly the third larval stage in cultures containing cells of Grace's *Aedes aegypti* line. These authors did not describe moulting in vitro and Weinstein (1970) suggested that the larvae produced in this culture system were probably late first stage organisms which had failed to moult completely. More recently Schiller et al. (1979) claimed to have obtained the development in vitro of a small number of *Onchocerca volvulus* microfilariae to the third and fourth larval stages.

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In an alternative approach to the cultivation of the arthropod stages of filariae, Taylor (1960) endeavoured to culture various stages of *Dirofilaria immitis* within malpighian tubules dissected from infected mosquitoes. The survival of the early stages (day 5–11) was generally poor, but second stage larvae (day 12) moulted to the third stage in some media. Weinstein (unpublished, in Weinstein, 1970) initiated a similar series of experiments with day 2 or day 4 larvae of *D. immitis* in malpighian tubules dissected from *Anopheles quadrimaculatus*. In medium NCTC-109, supplemented with serum and a gas phase of 5% $CO_2$, a small percentage of the larvae moulted to the second stage, but many badly stunted larvae were observed. Weinstein (unpublished, in Hansen and Hansen, 1978) later observed development in vitro to the third larval stage of day 1 larvae in malpighian tubules when these were cultured in medium containing 40% serum.

The purpose of this present study was to determine whether infective third stage larvae of *D. immitis* could indeed be produced in cultivation systems utilising organ cultures and, if so, to determine how the culture system could be simplified while still supporting larval development. The development of *D. immitis* larvae in malpighian tubules excised from mosquitoes 20–24 h post infection has been studied in a variety of tissue culture media, both insect and mammalian. The effect on larval development of including a feeder cell layer in the cultures, of differing the serum concentration of the medium and of altering the gas phase of culture has also been investigated.

## Materials and methods

### *Preparation of organs for culture*

Mosquitoes of the susceptible black-eyed Liverpool stock of *Aedes aegypti* were reared and maintained in an insectary at a temperature of 28°C ± 2°C and a relative humidity of 75–80%. Five to six days post-emergence, mosquitoes were membrane fed on *D. immitis* infected blood with a count of 100–150 microfilariae per 20$\mu$l of blood. Twenty to 24 h later, by which time the microfilariae have penetrated the cells of the malpighian tubules, the mosquitoes were killed and surface sterilised by a brief immersion in 70% ethanol. The mosquitoes were dissected in Hayes' saline (Hayes, 1953) containing 1,000 units of benzypenicillin, 1,000 $\mu$g of streptomycin (as 1,000 units/ml of 'Crystamycin', Glaxo Laboratories, Greenford, England) and 200 units of nystatin ('Mycostatin', Squibb, Liverpool) per ml and the malpighian tubules were transferred in groups of 4–6 to sterile plastic Petri dishes under the laminar flow cabinet. All subsequent experimental procedures were carried out using aseptic techniques. The tubules were washed in three changes of Hayes' saline containing antibiotics for 5–6 min each wash and were then inoculated individually into sterile glass Leighton tubes containing 2 ml of the appropriate culture medium.

Wherever possible the hind gut was left attached to the tubules as it was anticipated that the regular contractions of the gut might give an indication of the viability of the cultures.

### *Culture systems*

The following media were tested: 199, NCTC-135, RPMI-1640 (all obtained from Flow Laboratories, Irvine, Scotland), Grace's insect medium, Schneider's Drosophila medium, Leibovitz-15, modified as described by Pudney and Lanar (1977) (all obtained from Gibco Bio-cult,

Paisley, Scotland), and medium MM/MK (Varma and Pudney, 1969). All the media contained 2% Hepes Buffer (Flow Laboratories) and 200 units of benzylpenicillin, 200 $\mu$g of streptomycin and 50 units of nystatin per ml. The media were all supplemented with 20% heat inactivated foetal bovine serum (Flow Laboratories), except in those experiments in which the effect of increasing the serum concentration to 40% was investigated. With the exception of medium RPMI-1640 and Leibovitz-15 which were tested only with cells, all media were tested with and without a feeder layer of *Ae. malayensis* cells (Varma et al., 1974).

The cultures were incubated at 28° C in a gas phase of air, although in some experiements the effect of gassing the cultures with either 5% $CO_2$ in air or 90% $N_2$, 5% $O_2$, 5% $CO_2$, was investigated. The cultures were examined at regular intervals using an inverted microscope. The larvae of *D. immitis* develop to the second stage in approximately 6–8 days and to the infective stage in 12–13 days in *Ae. aegypti* (Liverpool) maintained at a temperature of 28° C ± 2° C and at a humidity of 75–80%. Cultures were generally terminated on day 7 post inoculation (i.e. day 8 of infection) and on day 10 or day 13 but some cultures were maintained for 16 days in vitro. *D. immitis* larvae recovered from the cultured tubules were fixed and mounted following the method of Wharton (1959) for more detailed examination and measurement. The larvae were classified into one of four groups according to their degree of development: microfilariae or intermediates (stages intermediate in development between microfilariae and sausage stages); sausage stage larvae; post-sausage stage larvae (larvae developing beyond the sausage stage but failing to moult to the second stage); second stage larvae.

## Results

*Cultures without a feeder cell layer*

In malpighian tubules cultured in MM/MK, NCTC-135, 199 and Grace's insect medium no development of the larvae of *D. immitis* occurred beyond the late first stage (or sausage stage) even after 13 days of culture. The sausage stage larvae recovered from tubules cultured in these media were normal in appearance and were equivalent to day 3–4 larvae in vivo. Increasing the serum content of each of the media to 40% or gassing the cultures with 5% $CO_2$ in air failed to stimulate further development of the larvae.

Over 30% of the larvae recovered from tubules cultured for 7 days in Schneider's Drosophila medium had developed to a stage beyond the sausage stage. The pooled results of five separate experiments carried out with Schneider's medium, with and without *Ae. malayensis* cells, are summarised in Table 1. In tubules dissected on day 7 post inoculation (or day 8 of infection) 30% of the recovered larvae were classified as post sausage stages and only 1% as second stage larvae, whereas in tubules dissected on day 10–13 of culture, 20% of the larvae were post sausage stages and 13% of the larvae had completed the moult to the second stage.

Post sausage stage larvae recovered from tubules cultured in Schneider's medium measured between 228–334 $\mu$m in length, 19–25 $\mu$m width at the head end and 36–44 $\mu$m in the region of the anal vesicle. These larvae were freely moving compared to sausage stage larvae, which show little active movement, the cuticle was frequently loosened at the head or at the tail end and the developing intestine could be clearly differentiated (Fig. 1).

Table 1. The development of *D. immitis* in malpighian tubules cultured in Schneider's Drosophilia medium with and without *Ae. malayensis* cells

| Medium | Cells | Day of dissection | Total number larvae recovered | % development to each stage | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Microfilariae/ intermediate | sausage stage | post-sausage stage | second stage | Mean number larvae/tubule | Number tubules dissected |
| Schneider's ..... | – | 7 | 115 | 15 | 54 | 30 | 1 | 9 | 13 |
| Schneider's ..... | + | 7 | 186 | 26 | 44 | 23 | 7 | 10 | 18 |
| Schneider's ..... | – | 10–13 | 67 | 21 | 46 | 20 | 13 | 4 | 15 |
| Schneider's ..... | + | 10–13 | 109 | 3 | 57 | 25 | 15 | 5 | 22 |

– = *Ae. malayensis* cells not included in cultures
+ = *Ae. malayensis* cells included in cultures

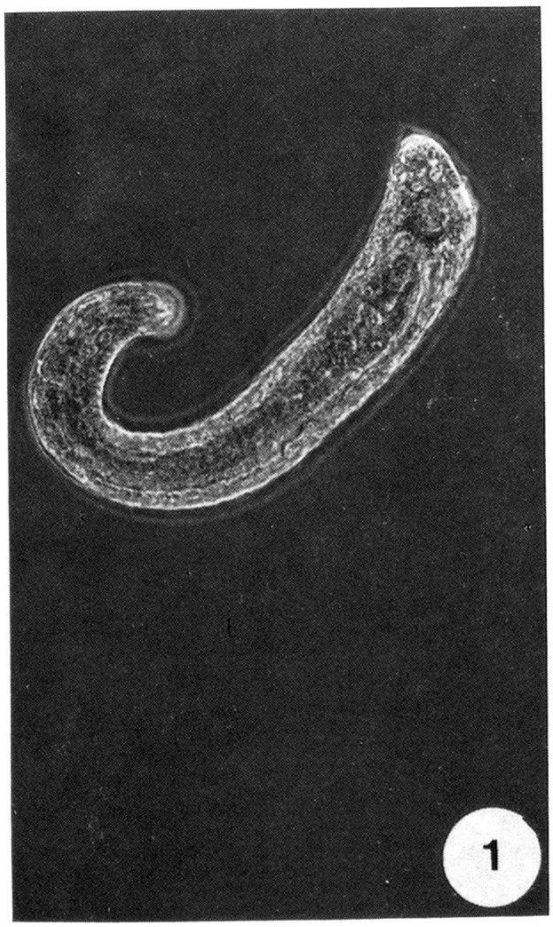


Fig. 1. Post sausage stage larva of *D. immitis* recovered from tubules after 7 days of culture in Schneider's medium plus *Ae. malayensis* cells. ×252.

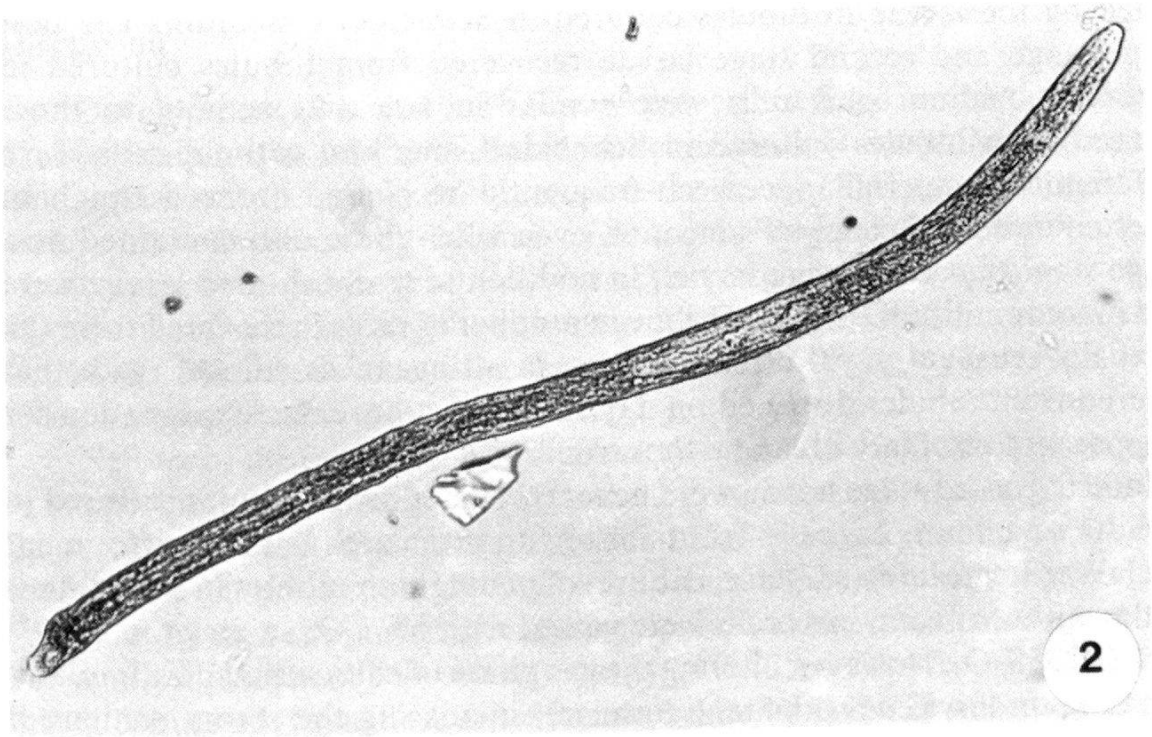


Fig. 2. Late second stage larva of *D. immitis* recovered from tubules after 13 days of culture in Schneider's medium without cells. ×175.

The second stage larvae produced in vitro were normal in appearance and were extremely active. These larvae measured between 315–714 $\mu$m in length, 19–25 $\mu$m width at the head end and 25–32 $\mu$m in the region of the anus (Fig. 2). Where second stage larvae or well-developed post sausage stages were recovered, these were invariably found grouped together at the swollen distal ends of the tubules. Development beyond the second stage has not been achieved even in tubules cultured in Schneider's medium for 14–16 days.

Medium RPMI-1640 and Leibovitz-15 were tested only in cultures which included a feeder layer of the *Ae. malayensis* cells.

*Cultures with a feeder cell layer*

No development of *D. immitis* larvae beyond the sausage stage occurred in tubules cultured in MM/MK, NCTC-135 or Grace's insect medium when *Ae. malayensis* cells were included in the cultures.

In tubules cultured for 7 days in medium RPMI-1640, 199 or Leibovitz-15 in the presence of *Ae. malayensis* cells, up to 25% of the larvae developed to the post sausage stage. However, development of the larvae did not proceed to the second stage even in tubules cultured for 10–13 days in these media.

In Schneider's Drosophila medium in the presence of *Ae. malayensis* cells, 30–40% of the larvae developed to a stage beyond the sausage stage. In some tubules cultured in Schneider's medium plus cells, second stage larvae were present by day 7 of culture, but a greater number of second stage larvae were generally recovered from tubules cultured for 10–13 days (see Table 1). The inclusion of a feeder cell layer did not enhance the degree of development attained by the larvae in tubules cultured in Schneider's medium. The post sausage stage and second stage larvae recovered from tubules cultured in Schneider's medium plus cells were similar in size and activity to those recovered from tubules cultured in Schneider's medium without cells. Live undifferentiated microfilariae were frequently recovered from malpighian tubules cultured in Schneider's medium even when these also contained post sausage stage or second stage larvae. In addition severely stunted larvae were found in some cultures (Fig. 3). The mean number of larvae recovered from the tubules dissected on day 7 of culture was, in all instances, almost twice that recovered from tubules dissected on day 10–13 of culture. No explanation for this apparent loss of larvae can be forwarded.

Infective third stage larvae were never recovered from tubules cultured in Schneider's medium plus cells. In attempting to stimulate further development of the larvae to the infective stage, cultures of malpighian tubules in Schneider's medium with *Ae. malayensis* cells were gassed with 5% $CO_2$ in air or with 90% $N_2$, 5% $CO_2$ 5% $O_2$. However, altering the gas phase of culture had no stimulatory effect upon larval development. Similarly increasing the serum content of Schneider's medium to 30% or to 40% also failed to stimulate development to the infective stage.

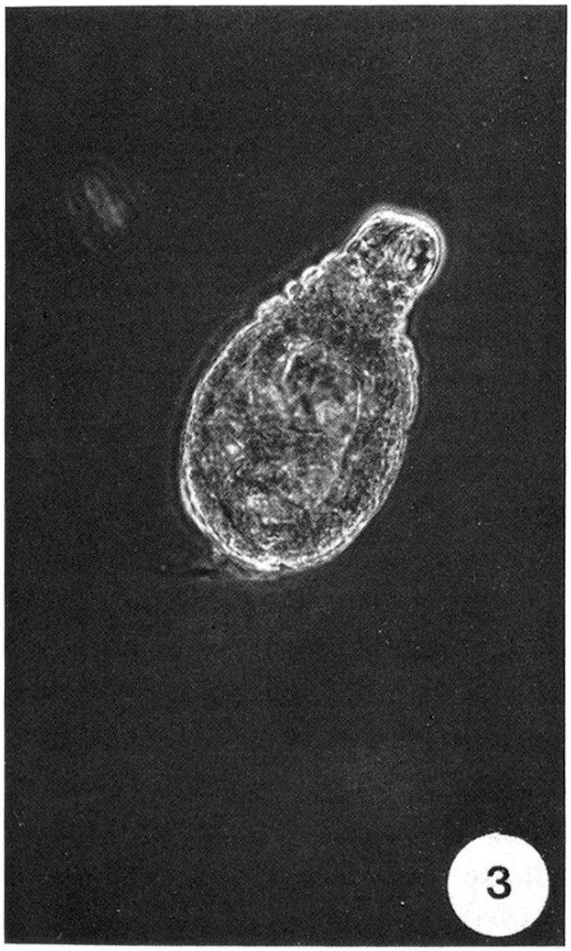


Fig. 3. Abnormal first stage larva of *D. immitis* recovered from tubules after 13 days of culture in Schneider's medium plus *Ae. malayensis* cells. ×252.

## Discussion

Development of *D. immitis* larvae to the second stage has been obtained in excised malpighian tubules cultured in Schneider's Drosophila medium containing 20% serum. In this culture system the presence of a feeder layer of *Ae. malayensis* cells did not significantly enhance the degree of development of the larvae. Infective third stage larvae were never recovered from tubules even after 16 days of culture. Gassing the cultures with either 5% $CO_2$ in air or with 90% $N_2$, 5% $O_2$, 5% $CO_2$ or increasing the serum content of the medium to 40% also failed to stimulate development to the $L_3$.

Neither of the other insect media tested, MM/MK or Grace's, was capable of supporting development of the larvae beyond the sausage stage. Similarly the results obtained with medium NCTC-135 were disappointing considering that this medium is almost identical to medium NCTC-109 used by Weinstein (1970) and which supported larval development to the infective stage. NCTC-109 is not available in this country but NCTC-135 differs from NCTC-109 in that it contains L-cysteine hydrochloride, while NCTC-135 does not. It is unlikely that the differences in results between this study and that of Weinstein can be attributed to the lack of L-cysteine in the medium. Previous experiments

with *D. immitis* demonstrated that the addition of L-cysteine to microfilarial cultures did not enhance the degree of larval development (Devaney and Howells, 1979). As with Schneider's medium, increasing the serum content of NCTC-135 to 40% or gassing the cultures with 5% $CO_2$ in air failed to stimulate further development of the larvae in this present study.

Medium RPMI-1640, 199, Leibovitz-15 were all capable of supporting larval development beyond the sausage stage when cells were included in the cultures, but the larvae failed to moult to the second stage. RPMI-1640 and Leibovitz-15 were not tested in cultures without cells, but in the case of medium 199 the presence of cells in the cultures appeared to have a stimulatory effect upon larval development; post sausage stage larvae were recovered from tubules cultured in 199 plus cells, but not from tubules cultured in 199 without cells.

Only Schneider's medium was capable of supporting significant development to the second larval stage, but it is unclear whether the results obtained with this medium were due to a direct effect of the medium upon the larvae (e.g. the provision of an essential metabolite) or were due to the improved viability of the tubules cultured in this medium. The maintenance of contractions of the hind gut in culture was an unreliable indicator of the viability of the tubules or of the larvae, for in some experiments in which the contractions stopped after a relatively short time in culture the larvae successfully developed to the second stage while in other instances no larval development occurred although the gut continued to contract.

In these experiments the malpighian tubules were excised from mosquitoes 20–24 h post infection. Although at this time the larvae within the tubules closely resemble microfilariae in their morphology, specific changes are occurring, divison of the G cell has been initiated and has already occurred in approximately 15% of the larvae. This cell division, or some other undetermined physiological change, which occurs within the first 24 h, appears to be a prerequisite for development of the larvae to the second stage in culture, for in those cultures initiated with infected tubules excised from mosquitoes at less than 24 h, larvae do not develop to the second stage (Devaney and Suliman, unpublished observation).

Infective larvae of *D. immitis* could not be produced by any of the culture techniques employed in this study, despite the use of organ cultures and of feeder cell layers. The results obtained here cannot be readily compared with those of Taylor (1960), Weinstein (1970) and Weinstein (unpublished, in Hansen and Hansen, 1978). The infected tubules employed by Taylor were excised from mosquitoes on the fifth day of infection, by which time the larvae would have undergone a significant degree of development in vivo. Weinstein (in Hansen and Hansen, 1978) did employ day 1 larvae in malpighian tubules excised from *Ae. aegypti* and obtained development to the infective stage, but inadequate details of this study were published to permit comparison. In a more

detailed earlier account (Weinstein, 1970) day 2 and day 4 larvae in malpighian tubules from *Anopheles quadrimaculatus* were employed but these developed only to the second stage in vitro. A serum level of 40% was found to be necessary for production of infective larvae by Weinstein (in Hansen and Hansen, 1978) but in the present study increasing the serum concentration of the medium to 40% failed to stimulate further development of the larvae.

Following the report of Weinstein (in Hansen and Hansen, 1978) it was expected that the cultures of infected tubules would readily support larval development to the infective stage in vitro, and attempts could then be made to analyse and simplify the culture system. Infective larvae could not be obtained in such organ cultures but they did support larval development to the second stage, a significant advance over most other culture systems employing extracellular larvae, which permit development only to the sausage stage. Since the intracellular milieu of the tubule cell must provide the appropriate physical conditions for larval development, both in vivo and in vitro, a greater degree of larval development in vitro will presumably result from improvements in the liquid culture medium employed. Such modifications, however, would have to be based upon an empirical scheme rather than a rational assessment of the metabolic requirements of the insect or nematode tissues, since little is known of the nutritional or other requirements of either the tubules or the worms. The original objective of this study, that is, to obtain larval development in a complex system and then to attempt a progressive simplification of the culture system is thus of dubious practicality. Even more difficult is the further extension of the concept of organ culture to the cultivation of the filarial species of medical importance. The arthropod stages of *Wuchereria bancrofti*, *Brugia malayi* and *Onchocerca volvulus* develop in the thoracic flight muscle and these tissues present a significantly greater obstacle to successful organ culture than do malpighian tubules.

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