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In vitro development of third- and fourth-stage larvae of *Dirofilaria immitis*: comparison of basal culture media, serum levels and possible serum substitutes

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Summary

In vitro development and survival of third-stage larvae of *Dirofilaria immitis* were compared in four different culture media and in the presence of varying concentrations of four different medium supplements. Motility and the incidence of third- to fourth-stage molting were used as criteria for evaluating different culture conditions. No significant differences in either motility or molting response were detected between larvae cultured in NCTC-135, F12(K), CMRL 1066 or Dulbecco's Modified Eagle's Medium. Fetal calf serum enhanced development and survival of the cultured larvae in dose-dependent fashion. Its effects were maximal at a concentration of 20 percent of the total medium volume. Addition of a commercial medium supplement, NuSerum, also gave a dose-related increase in larval development and viability. The activity of NuSerum in this respect was comparable to that of fetal calf serum. The tripeptide glycylhistidyllysine and bovine serum albumin, fraction V both failed to stimulate development of third-stage *D. immitis* larvae in vitro.

Key words: *Dirofilaria immitis*; Filarioidea; in vitro cultivation; infective larva; molt.

Introduction

Molting and limited growth of third-stage larval *Dirofilaria immitis* has been observed in a number of in vitro culture systems. Serum-supplemented media with additives such as canine tissue explants (Taylor, 1960), whole canine blood (Yoeli et al., 1964) and various mammalian cell lines (Wong et al., 1982)

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have yielded positive results. Similar development of larval *D. immitis* occurred under cell-free conditions in the medium NCTC-109 supplemented with 10 percent human or equine serum (Sawyer, 1963, 1965). A reduced molting response and rapid degeneration of larvae occurred in serum-free NCTC-109 (Sawyer, 1965).

In vitro systems are viewed as a means of producing stage-specific somatic and excretory/secretory antigens free of complicating immunological factors from the host (WHO, 1981). Collection of antigens from cultured filarial larvae would be greatly facilitated if the use of non-defined, high molecular weight medium components could be minimized or, if possible, eliminated entirely. To date, however, a chemically defined, low molecular weight medium suitable for cultivation of infective filarial larvae has not been identified. The present paper presents findings on the molting response and survivorship of third-stage larval *D. immitis* cultured in different basal media, with different concentrations of fetal calf serum and in the presence of three potential serum substitutes.

Materials and Methods

Third-stage larvae of *D. immitis* were obtained from infected female *Aedes aegypti* of the Liverpool selected strain (Macdonald, 1962; Macdonald and Ramachandran, 1965). Mosquitoes were infected with *D. immitis* by feeding on heparinized dog blood containing microfilariae at a concentration of approximately 100 parasites per ml. The microfilarial suspension was fed to the mosquitoes using an artificial membrane feeding apparatus (Rutledge et al., 1964).

Fifteen days after infection, mosquitoes were anesthetized by placing on crushed ice. The insects were then surface sterilized by quickly emerging in 95% ethanol and then washing in one percent aqueous benzalkonium chloride (Calbiochem-Behring, San Diego, California, USA) for two to three minutes at room temperature. Aseptic technique was observed hereafter. Mosquitoes were then rinsed in two changes of Moscona's saline and placed in 60 mm petri dishes containing 10 ml of dissection medium. Dissection medium consisted of NCTC-135 (Gibco Laboratories, Grand Island, New York, USA) or F12(K) (KC Biological, Lenexa, Kansas, USA) containing 10 percent fetal calf serum (FCS, Flow Laboratories, Dublin, Virginia, USA), 0.025 M HEPES (Research Organics Inc., Cleveland, Ohio, USA), gentamicin (100 µg/ml, Schering Corp., Kenilworth, New Jersey, USA), sulfadiazine (50 µg/ml), trimethoprim (10 µg/ml) added as Tribriassin (Burroughs Wellcome Co., Kansas City, Missouri, USA) and mycostatin (20 U/ml, Gibco Laboratories, Grand Island, New York, USA).

Heads and mouthparts of the infected mosquitoes were then removed and teased thoroughly with insect pins. The emerging larvae were counted, collected with pasteur pipets and pooled in a 15 ml centrifuge tube. Pooled larvae were concentrated by centrifugation at $900\times g$ for five minutes, and the resulting pellet was washed twice with 15 ml of NCTC-135 or F12(K) containing 0.025 M HEPES. In the early phases of this study (medium comparisons) third-stage larvae were treated for five minutes prior to the final washes with a one percent solution of commercial chlorine bleach in NCTC-135 as a further precaution against microbial contamination. A similar technique was used by Chen and Howells (1979). However, this treatment proved ineffective in combating bacterial contamination, and it was abandoned in the latter phases of the work. Washed larvae were resuspended in the test culture media at a concentration of 30 to 50 larvae per ml and transferred to flat-sided plastic culture tubes (Gibco Laboratories, Grand Island, New York, USA). The test culture media contained antibiotics at the concentrations given for the dissection medium. Cultures were maintained at 37° C and 95 percent relative humidity with a gas phase of five percent CO₂ in air. The

pH of all cultures was 7.2. Cultures were observed daily and media were changed at 48-hour intervals.

Development and viability of cultured larvae was evaluated on the basis of motility and frequency of third- to fourth-stage larval molting. The number of parasites completing the third molt in a given culture was determined by counting cast third-stage cuticulae. The number of molts was expressed as a percentage of the total number of larvae in the culture. Motility was scored on a three-point scale with 0 indicating larvae which were inactive or damaged, 1 indicating sluggish or intermittent activity and 2 indicating constant, vigorous serpentine movements. Mean activity scores were calculated for all larvae in a given culture.

Data from medium comparisons were analyzed by one-way analysis of variance. Where linear comparisons were suggested by the data, the conservative method of Scheffe' (1959) was employed. Median lengths and 95% confidence intervals were determined for larvae before and after in vitro cultivation and were compared using the Mann-Whitney rank-sum test (Snedecor and Cochran, 1967).

Results

Behavior of cultured larvae. As indicated by trends in motility scores over time (Fig. 1a) parasites which were highly motile at culture inoculation became lethargic during the first 24 hours in vitro. Normal vigorous motility patterns resumed during the next 24 to 48 hours. Molting commenced some 48 hours after inoculation and was complete by the fourth day in culture (Fig. 1b). Parasites which failed to molt at this time became vacuolate and inactive. This observation is substantiated by the decreasing trend in motility scores recorded after the third day in culture (Fig. 1a). A slight downward trend in cumulative percent molting was noted after the fifth day in vitro. During this period median length of the larvae increased from 1044 μm (1008–1080; $n = 50$) to 1278 μm (1260–1332; $n = 28$). This increase in length was highly significant ($p < 0.001$).

Comparison of basal culture media. Four standard cell culture media, NCTC-135, CMRL-1066 (Gibco Laboratories, Grand Island, New York, USA), Ham's F12(K) and Dulbecco's Modified Eagle's Medium (DMEM, KC Biological, Lenexa, Kansas, USA), were tested for their ability to support in vitro molting of third-stage *D. immitis* larvae. NCTC-135 and CMRL 1066 were chosen for testing because these media or similar formulations (NCTC-109) have been used previously for cultivation of third-stage larvae of *D. immitis* (Sawyer, 1963, 1965; Yoeli et al., 1964). F12(K) was chosen because it has demonstrated an excellent capability to support the differentiation of microfilariae of *D. immitis* to the first larval stage in vitro (authors' unpublished observations) and DMEM because it has been used successfully to culture larvae of *Ascaris suum* from the third to the fourth stage in vitro (Urban and Douvres, 1981). Each of the test media was supplemented with 10% fetal calf serum. The results in Table 1 show that all of the media supported molting of an average of at least 50 percent of the parasites. NCTC-135 gave the highest mean percentage molting. Variation in the means attributable to the different culture media, however, was not significant at the five percent level. A comparison of mean percent molting in NCTC-135 and F12(K) which was suggested by the data also

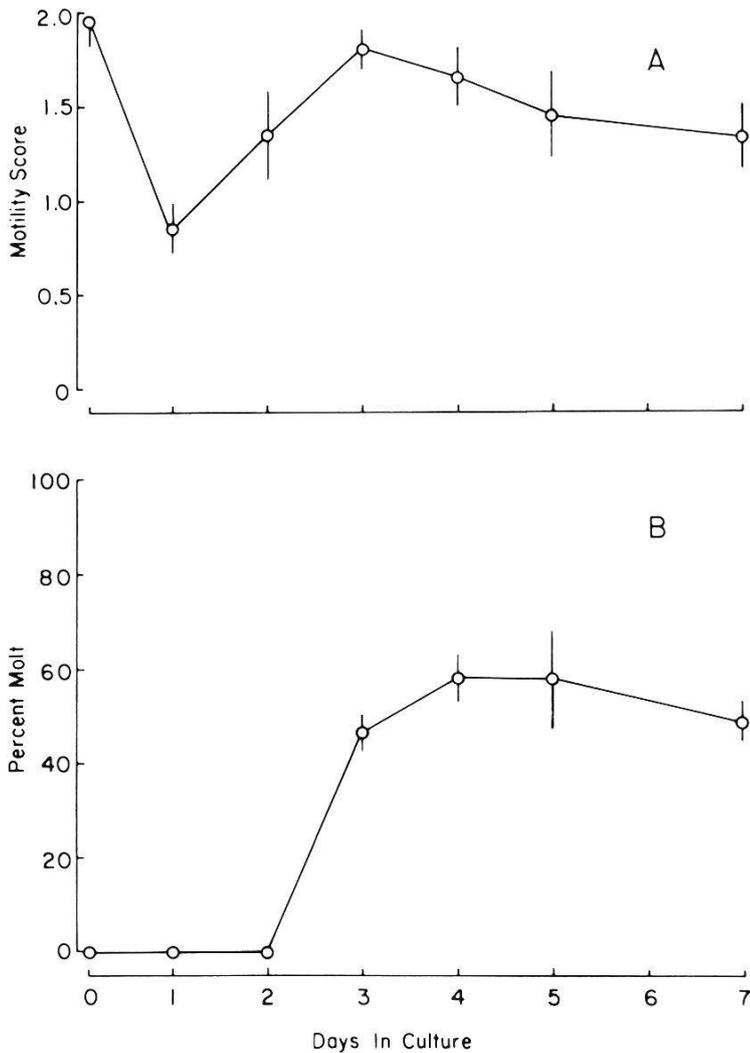


Fig. 1. Motility score (A) and cumulative percent molting (B) of third-stage larval *Dirofilaria immitis* during the first seven days of in vitro culture. Larvae were cultured in F12(K) with 10% fetal calf serum. Each point is the mean of counts from three separate cultures with at least 50 larvae per culture. Vertical bars represent the standard error of the mean.

Table 1. Molting and motility scores for third-stage larval *D. immitis* cultured in different basal media¹

Medium ²	No. larvae observed ³	Mean (± SE) percent molt	Mean (± SE) motility score
NCTC-135	225	$p^4 > 0.05 \left\{ \begin{array}{l} 74.5 \pm 6.3 \\ 56.5 \pm 4.4 \\ 62.8 \pm 7.5 \\ 55.8 \pm 11.6 \end{array} \right\} p > 0.1$	$\left. \begin{array}{l} 1.43 \pm 0.17 \\ 1.42 \pm 0.11 \\ 1.55 \pm 0.08 \\ 1.54 \pm 0.08 \end{array} \right\} p > 0.1$
F12(K)	216		
CMRL-1066	208		
DMEM	193		

¹ Molting and motility were assessed seven days after culture inoculation.

² All media were supplemented with 10 percent fetal calf serum.

³ Numbers of larvae represent totals observed in three separate experiments.

⁴ Values of p greater than 0.05 are considered indicative of a non-significant result.

gave a non-significant result. Similarly, mean motility scores for parasites cultured in the four test media were not significantly different. Although NCTC-135 gave the highest percent molting response in the medium comparisons, it gave highly variable results in subsequent experiments. Efforts to trace the source of this variability were not successful. Medium F12(K) gave much more consistent results overall and was chosen as a basal medium for trials involving various medium supplements.

Effects of medium supplements on development of cultured larvae. Fetal calf serum and three serum substitutes were tested for enhancing effects on motility and molting response of third-stage larvae cultured in medium F12(K). All fetal calf serum was from a common lot (Flow Laboratories, lot No. 29111015) and was heat inactivated. Fetal calf serum (FCS), at concentrations of five to 40 percent (v/v) promoted molting and a high rate of motility (Fig. 2a). The frequency of molting increased in proportion to the concentration of FCS up to 20 percent. Increasing the concentration of FCS to 40 percent appeared to have no further enhancing effect on the molting response. Motility scores in all FCS-supplemented media were relatively high, and they appeared to be independent of serum concentration. The incidence of molting in non-supplemented F12(K) was relatively low. Larvae cultured under serum-free conditions usually became moribund by the fifth day in culture as indicated by low motility scores (Figs. 2a–d). Molting frequencies and motility scores recorded for larvae in serum-free cultures often varied significantly from one experiment to another as illustrated by Fig. 2b and 2d.

Addition of the tripeptide glycyLhistidyllysine (GHL, Collaborative Research, Waltham, Massachusetts, USA) to medium F12(K) at concentrations ranging one to four μg per ml had no apparent effect on the molting response of cultured parasites (Fig. 2b). Motility scores for parasites cultured in the presence of GHL were uniformly lower than those obtained with parasites cultured in serum-supplemented medium. There was, however, a slight increase in motility in response to increasing GHL concentration.

The effects of a commercially prepared medium supplement, NuSerum (Collaborative Research, Waltham, Massachusetts, USA) on molting and motility were similar to those for FCS over the same range of concentrations (Fig. 2c). Molting frequency increased in dose dependent fashion as the concentration of NuSerum increased from 0 to 10 percent of the medium volume. This response leveled off at concentrations above 10 percent. Motility scores were independent of NuSerum concentration at the levels tested, and they were markedly higher than those recorded for parasites in non-supplemented medium.

Finally, bovine serum albumin, fraction V (BSA, Sigma, St. Louis, Missouri, USA) was added to medium F12(K) at concentrations ranging from 1.25 to 5.00 mg/ml. There was no beneficial effect of BSA on molting or motility of the parasites (Fig. 2d).

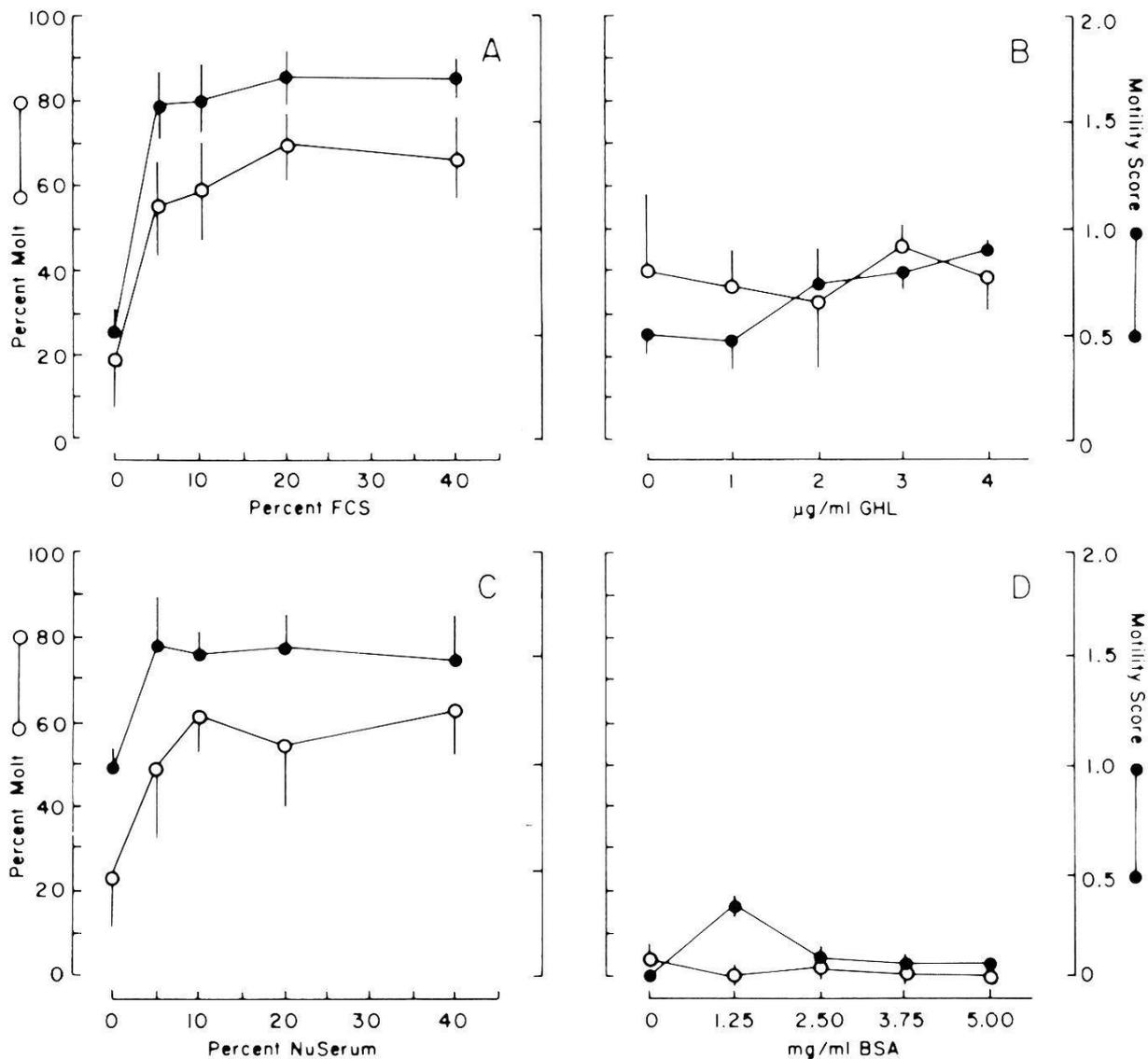


Fig. 2. Motility score (●) and percent molting (○) of third-stage larval *Dirofilaria immitis* cultured in the presence of increasing concentrations of medium supplements. Supplements tested included fetal calf serum (A), glycyhistidyllysine (B), NuSerum (C) and bovine serum albumin fraction V (D). Basal medium in all tests was F12(K). Molting response and motility were evaluated five days after culture inoculation. Each point is the mean of counts from three separate cultures with at least 30 larvae per culture. Vertical bars represent the standard error of the mean.

Discussion

The findings presented here support earlier observations (Sawyer, 1963, 1965) that mosquito-derived third-stage larvae of *Dirofilaria immitis* molt to the fourth stage in cell-free in vitro culture. Molting of the larvae began on the third day in culture and was complete by the fourth day. This observation is in agreement with other studies which indicate that *D. immitis* initiates the third molt in vitro some two to three days after culture inoculation (Sawyer, 1963, 1965). Studies of early development in the dog indicate that the third molt may be less

synchronous in vivo occurring over a period of three to 12 days after inoculation (Orihel, 1961; Kotani and Powers, 1982).

An anomalous decrease in cumulative percent molting on days five and seven was observed in the present study. This inconsistency may be an artifact of the technique of enumerating molts by counting cast larval cuticulae. Inadvertent removal of cast cuticulae on days four and six after inoculation could account for erroneous low counts.

The motility scores recorded during the first five days in vitro clearly indicate a lethargus prior to the molt. A similar lethargus was reported by Sawyer (1963) for *D. immitis* third-stage larvae cultured in NCTC-109. The increase in the size of cultured larvae in the present study is consistent with previous observations of early growth in vivo (Orihel, 1961; Kotani and Powers, 1982) and in vitro (Yoeli et al., 1964).

The results of the medium trials indicating a high incidence of molting in four different basal culture media are consistent with reports of molting by third-stage larvae of *D. immitis* under a wide range of in vitro conditions (Taylor, 1960; Yoeli et al., 1964; Sawyer, 1965; Wong, 1982). Medium F12(K) was chosen for further study because it yielded the most consistent overall results.

Fetal calf serum, at concentrations as low as five percent, gave a marked improvement in the molting response and motility of the parasites. It appears that in medium F12(K) maximum viability is achieved with FCS at a concentration of 20%. A slight decrease in the molting response at 40% FCS may indicate either a toxic effect of this additive or the dilution of some essential constituent of the basal medium not present in the serum.

Glycylhistidyllysine (GHL), a plasma-derived tripeptide, has been shown to stimulate growth and enhance viability in numerous types of cultured cells and organs (Pickhart, 1981). This tripeptide is thought to act as a transport factor for transition metals such as copper and is viewed as a means of reducing or eliminating the serum requirements of various in vitro culture systems (Pickhart, 1981). Stromberg et al. (1977) used GHL at a concentration of 14 ng/ml to replace porcine serum in a culture system which supported third- to fourth-stage molting of larval *Ascaris suum*. The resulting chemically defined culture system allowed the isolation of a protective excretory/secretory antigen from this nematode larva (Stromberg and Soulsby, 1977; Stromberg, 1979). A similar level of GHL (20 ng/ml) failed to replace serum in a culture system for third- to fourth-stage larvae of the filariid *Dipetalonema viteae* (Tanner, 1981). Nelson et al. (1982) reported molting of rat-primed third-stage larvae of *Litomosoides carinii* in medium L-15 supplemented with 2 $\mu\text{g}/\text{ml}$ GHL. The molting response in the presence of GHL was inconsistent compared to results obtained with serum supplemented L-15. Cultivation in serum free L-15 was not attempted. In the present study GHL at one to four $\mu\text{g}/\text{ml}$ did not stimulate molting of larval *D. immitis* and its effect on motility was negligible.

NuSerum is a commercially prepared medium supplement containing

growth, transport and cell attachment factors, hormones and nondefined serum products. The present findings indicate that it is very similar to fetal calf serum in its enhancing effects on in vitro development and motility of cultured third- and fourth-stage larvae of *D. immitis*. The total protein concentration of the NuSerum lot used in this study was 13.2 mg/ml, less than one half the average protein content of fetal bovine serum (Price and Gregory, 1982). The reduced protein levels in NuSerum supplemented media may facilitate isolation of excretory/secretory products from cultured parasites.

Bovine serum albumin, fraction V, at concentrations ranging from 1.25 to 5.00 mg/ml failed to support molting or survival of the larval *D. immitis*. This concentration range roughly approximates albumin levels found in fetal calf serum (Price and Gregory, 1982). It would appear therefore, that components other than albumin are responsible for the growth-enhancing effects of FCS.

Developmental capacity and survivorship of larval *D. immitis* were both depressed in serum-free culture as indicated by low molting frequencies and motility scores respectively. Under serum-free conditions these parameters often varied to a great extent from one experiment to another (e.g. Figs. 2b and 2d). The reason for this inconsistency is uncertain. One possible explanation is subtle variation in the viability or fitness of larvae which are initially placed in culture. Such variation in larval fitness, while inapparent in serum-supplemented media, might be expressed in the suboptimal culture environment represented by serum-free medium. Variability in intrinsic larval fitness might stem from sources such as differential carry-over of nutrient reserves from the vector or stress incurred during isolation from the insect host.

The levels of gentamicin sulfate and mycostatin added to the test culture media in the present study correspond to concentrations generally recommended for in vitro cultivation of animal cells (Schaffner, 1979) and parasitic nematodes (Hansen and Hansen, 1978). At these concentrations gentamicin and mycostatin have shown no detectable cytotoxic effects in vitro (Schaffner, 1979). The sulfadiazine/trimethoprim formulation used in the present study has not, to the authors' knowledge, been employed in the in vitro cultivation of any parasite. Its addition was necessitated by frequent contamination of larval preparations with a gentamicin-resistant bacterium, *Achromobacter xylosoxans*. *Achromobacter* sp. is commonly found in the midgut of *Aedes aegypti* (Westreich and Chao, 1963). As discussed by Hansen and Hansen (1978), the possible effects of antibiotics and antimycotics on the development and survival of parasitic nematodes in vitro have not been established previously, and these potential effects were not assessed in the experiments reported here. It is possible that the antibiotics employed in the present study exert an inhibitory effect on filarial morphogenesis and may account for the failure of a portion of the larval *D. immitis* to develop in vitro. A systematic investigation of the influence of these agents on filarial development in vitro is called for.

In summary, the medium supplement NuSerum allows successful in vitro

cultivation of third- and fourth-stage larval *D. immitis* in a medium with reduced overall protein content. However, a chemically defined culture medium for these parasites has yet to be developed.

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