Mermithid Nematodes: In Vitro Culture Attempts[1]

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Abstract: Few attempts at in vitro culture of mermithids have been undertaken. The various methods used to initiate cultures are described. The capacity of a range of media to promote growth and development of the nematodes has been evaluated and current approaches to in vitro outlined. *Key words: Hydromermis conopophaga, Isomermis* sp., in vitro, in vivo, mass production, mermithid nematodes, *Neomesomermis flumenalis, Romanomermis culicivorax,* tissue culture, tissue culture media.

The widespread availability of mermithids as practical and economically competitive insect control agents depends upon the establishment of in vitro culture methods for their mass cultivation.

Culture in vivo is the only mass production method presently available for mermithids, but even this, in North America, is limited to *Romanomermis culicivorax* (20). Other mermithids, seriously considered for mosquito control have been maintained in the laboratory, but difficulties have been encountered in scaling up to the mass production level. These have included the lack of an easily maintained laboratory host (6), lack of synchronous egg hatch (5,19), and low male nematode production (19).

Blackfly mermithids have not been cultured in vivo because until recently it was not possible to cycle blackflies on a continuous basis. However, in 1978 a laboratory colony of *Simulium decorum* was established (32). This will allow challenge of the blackfly with naturally occurring or other blackfly parasitic mermithids for the possible establishment of an in vivo culture.

In vivo culture has an essential role to play in providing researchers with material for in vitro culture studies and for investigations of mermithid biology and physiology essential to their applications in control programs. It cannot, however, be considered as a long-term mass production method. The production costs are high, as the process involves rearing a host and this is labor intensive. The cost of the product will be correspondingly high and its use restricted.

By contrast, in vitro production methods offer benefits in the form of reduced costs for the production of a standard, stable, and viable entity. Development of an in vitro mass production method, however, must be viewed as a long-term project (leading from laboratory through pilot plant technology to commercial undertaking) and one that is capital intensive.

In vitro culture has only been attempted with a few species of mermithids: the mosquito parasite, *Romanomermis culicivorax,* (3,4,15,26,30); to a lesser extent, the blackfly parasitic mermithids, *Neomesomermis flumenalis* (3,14) and *Isomermis* sp. (3); and *Hydromermis conopophaga* from larval midges (21).

Compared to the time spent in the free-living environment, mermithids parasitic in biting flies spend a relatively short period in the hemocoel of their hosts. This parasitic phase, which varies in duration between species, can be conveniently divided into three stages for the purpose of assessing the capacity of culture media to promote growth and development. In the first stage, soon after entering the host, the long, slender nematode ceases rapid movement and becomes thicker and more granular as it adapts from its previous aquatic environment to the insect hemocoel. In the second stage the nematode casts its cuticle and its stylet. The third stage is marked by an extended period of enormous growth and concomitant development of the stichocytes, the trophosome (which becomes packed with stored nutrients), and the genital primordia. Thus, at the end of the parasitic phase, when the nematode finally emerges from its host, it is physiologically and structurally equipped to resume a free-living existence in water.

Culture of a mermithid through its parasitic phase in vitro necessitates the formulation of a culture medium which duplicates the environment provided by the host

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hemocoel and supports growth and development. Mermithids are not typical nematodes: lacking a functional gut, they must rely on transcuticular uptake to obtain their nutritional requirements (23,24,29). Until recently, relatively little has been understood of the complex relationships which exist between host and parasite. Attempts at in vitro culture have focused on the use of synthetic media and tissue culture techniques which have been adapted as new information on the physiological aspects of the host-parasite relationships have become available. Both successful and unsuccessful procedures and results are reported here and unless otherwise indicated, reflect the author's unpublished data.

HISTORICAL REVIEW

A range of commercially available tissue culture media has been used in attempts to delineate the nutritional requirements of *R. culicivorax* and assess the physical parameters necessary for its development. Sterilized eggs or preparasites were used to initiate cultures. Sterility must be maintained throughout the culture period, since the introduction of a contaminant, especially one that is slow growing, can give misleading results. This is especially critical in the early stages of culture.

Roberts and Van Leuken (26) found that preparasites of *R. culicivorax* hatched from eggs surface sterilized with sodium hypochlorite did not grow but died in less than a week when cultivated in the following: Mitsushashi and Maramorosch's leafhopper medium, modified Grace's medium (33), Schneider's Drosophilia medium plus fetal bovine serum (FBS) (4:1), Wolf and Quinby's amphibian culture medium, mycoplasma broth medium, *Caenorhabditis briggsae* medium, and water. Myers (15) pointed out that when cultures are initiated with axenized eggs, the cast cuticle from the molt, which normally occurs in the egg, is often seen floating in the media. This may be mistaken for the later parasitic molt. Myers (15) also reported that *C. briggsae,* Mitsuhashi and Maramorosch, and CMRL 1066 failed to promote growth; in Schneider's plus FBS (4:1) cell proliferation was observed and many of the mermithids burst. Growth did occur in Schneider's plus heat inactivated fetal calf serum (HIFCS) (4:1), but most nematodes did not grow evenly and their cuticles became bumpy. In addition, horse and chicken sera had growth promoting properties, but human, turkey, rabbit, porcine, and sheep sera did not. Neither could the fetal calf serum (FCS) be replaced by chicken embryo, beef embryo, or yeast extract. Most growth was eventually obtained in Grace's medium plus FCS (4:1). Myers included an axenizing agent in his cultures.

In contrast, Sanders et al. (30) inoculated their experimental media with preparasites axenized by passage through an antibiotic solution. In Schneider's Drosophila medium with 10% FCS at pH 6.5–7.0 and 25 C, nematodes developed through the first stage and increased in size, although the growth rate was 1/2–1/3 that of the nematode in vivo. No molt was seen in the culture medium; however, the stylet was reduced. The stichocytes and trophosome became more pronounced, indicating the onset of the third stage, but no gonadial development was observed. The medium was withdrawn and replenished during these incubations. Unfortunately, a distorted, lumpy nematode was depicted by Sanders et al. (30). This is the form of a nematode frequently encountered in unsuitable media. Such a nematode does not usually survive and cannot be considered a stage in development.

In view of the increased growth response obtained after introducing sterilized preparasites into a culture medium (30), compared with that achieved when sodium hypochlorite sterilized eggs were used to initiate cultures (15,26), the effects of axenizing agents on the viability of treated eggs and preparasites was assessed. Embryonated eggs were sterilized by single or multiple washes in 0.05% sodium hypochlorite. Egg hatches of 80% and 70–75% preparasite survival were obtained from eggs exposed once for a period not exceeding 30 min. Extended or repeated exposure reduced hatch to 20–25% and survival to 5%. The methods subsequently adopted involved individual sterilization of preparasites by passage through penicillin, streptomycin, fungizone, and kanamycin (PSFK) followed by washes in sterile distilled water

(4,11). This method, in contrast to that used for egg sterilization, ensured that all nematodes were in good condition at the time of placement in the culture medium. Although mass sterilization of preparasites was attempted by centrifugation for short periods in PSFK, individual sterilization, albeit time consuming, proved to be the more reliable method.

Culture of *R. culicivorax* was attempted in previously listed media plus medium 199, NCTC 109, and NCTC 135 in various ratios with water but no improvement was found over results already reported. Particular attention was paid to the use of media which had shown some promise: combinations of Grace's and/or Schneider's with a range of concentrations of chick embryo extract, FBS, and FCS. Axenizing agents were not included in media formulation, as their presence added another variable to the system. Growth occurred in these media to varying degrees, but the most successful results were obtained using Grace's containing 10% HIFCS at 26 ± 1 C with osmotic pressure adjusted to 240 mOsm and pH 6.4–6.5. In this medium the nematodes developed through the first stage. Following this the stylet was lost and, although no cast cuticle was observed, the nematodes continued to grow and develop as if in stage three. In fact, the cuticular molt is often difficult to detect as it occurs by a gradual sloughing off of the thin cuticle, parts of which can sometimes be seen trailing behind the nematode (22). This is in contrast to the more common nematode molt in which the cast cuticle slips off the nematode relatively intact. When it appeared likely that a complete molt had occurred in the medium but had gone undetected, a method was devised whereby mermithids which had passed this critical phase in vivo were dissected out of sterilized hosts into saline and incubated in the culture medium as before. The main difficulty occurred in the transference of newly molted nematodes (approx. 22 h after infection in *Aedes aegypti* at 25–27 C) into the medium, as at this time they are soft and sticky. As a result, only a few transferences were made; however, all subsequently developed to the final stage reached by nematodes introduced into the same medium as sterilized preparasites. It was concluded therefore, that a molt had occurred during the original incubations. Slow growth of the nematodes occurred over a 6-wk period during which the medium was changed. Eventually, gonadial development of females occurred, but storage material in the trophosome was lacking (4). The production of all female nematodes, which were in a nutritionally depleted state, does not fit with commonly held views on sex determination in mermithids (2,10,17, 18) and has yet to be explained. According to Ittycheriah et al. (13), the predominant storage metabolites in the trophosome of *R. culicivorax* are lipids and glycogen, with proteins playing a minor role. The lipid fraction is composed of triacylglycerols, phospholipids, free sterols, and sterol esters (8,9). In an attempt to improve transcuticular uptake and subsequent storage by the trophosome, recent research in this laboratory has been directed toward the incorporation of fatty acids into the culture medium. The nutritional state of the trophosome has been improved and development time of *R. culicivorax* reduced by the addition of myristic acid, squalene, and cholesterol.

Physical parameters are important components of a successful culture medium. The most suitable temperature for culture of *R. culicivorax* is 25–26 C, and the nematodes survive in the pH range 6.4–7.1. Media should be adjusted to an osmotic pressure comparable to that of the host haemolymph, while media volume, number of nematodes, and ionic ratios in the medium may be significant factors and should be taken into consideration. The effect of the gas phase on nematodes in culture has not been investigated thoroughly. Experimental gassing of the medium used by Finney with nitrogen or carbon dioxide did not improve rate or amount of development of the nematodes significantly. Sanders et al. (30) suggested that some solid matter may be necessary for culture of *R. culicivorax*. Cultures incorporating the more successful media mentioned above over nutrient agar slants were set up. The use of the solid-liquid medium did not improve results.

Roberts and Van Leuken (26) gave the first indication that tissue culture could be

used for culturing *R. culicivorax*. They incubated preparasites either in direct contact with insect tissue culture cells or separated from the cells by a membrane. The media used to culture the insect tissue were Schneider's and Mitsuhashi's and Maramorosch's; the cell lines were Singh's *Aedes aegypti* and Hsu's *Culex quinquefasciatus*. Moderate but slow growth was obtained. The authors noted that neither addition of sterols nor substitution of protein hydrolysates for sera improved growth. At this laboratory, limited growth of *R. culicivorax* has also been observed after introducing preparasites into an *Ae. albopictus* cell line.

There is evidence to suggest that mermithids utilize nutrients available within the host fat body (7) by inducing metabolic changes within the fat body tissue (7) and/or by hydrolyzing it through secretion of digestive enzymes (27,28). This theory, if substantiated, suggests that a fat body culture may be a suitable vehicle for nematode culture. A system has been devised at the research Unit on Vector Pathology based on Oberlander's (16) and Richman and Oberlander's (25) *Galleria mellonella* fat body culture method utilizing modified Grace's tissue culture medium. Insect nematodes can be incorporated into this medium. Research using this technique for culture of *R. culicivorax* is continuing.

It is possible to store blackfly parasitic mermithid eggs, preparasites or gravid females derived from field collected material, thus extending the period of availability of these mermithids for laboratory experimentation. However, the fact that these mermithids are not readily available on a year-round basis has hampered attempts to culture them in vitro.

Myers (14) incubated sterilized embryonated eggs and preparasites of *N. flumenalis* in a range of commercially available media at 15 C and achieved some nematode growth in Grace's tissue culture medium. Finney (3) used primarily sterilized preparasites of this nematode which had been stored previously at 5 C in tap water. Incubation in a combination of Schneider's Drosophila medium and Grace's tissue medium modified by the addition of FCS at 10 C resulted in some growth and development. The same medium promoted limited growth of preparasites of *Isomermis* sp. in cultures initiated from sterilized eggs prestored at 10 C. A molt was not observed during this series of experiments. The only particular considerations in initiating these cultures were to the suitable physical parameters: temperature and osmotic pressure. Otherwise experimental procedures and choice of medium were based on the attempts to culture *R. culicivorax*. However, it is becoming increasingly obvious that the media requirements for blackfly mermithid culture will not be the same as for *R. culicivorax* (8,9) and this will affect future media choice.

At least two attempts were made to establish blackfly cell lines with the intention of studying the host-parasite relationships and the initiation of a mermithid culture (1,31). However, as yet there is no data on attempts to pass mermithids through them.

The *Galleria* fat body technique has been adapted to accommodate *N. flumenalis* and this line of investigation continues, subject to the availability of the mermithid.

A single attempt was made to culture the preparasitic stage of *Hydromermis conopophaga* (21). The nematodes were surface sterilized in a 0.25% hyamine solution for 10 sec. A range of culture media were used: bovine serum, chick embryo extract, rabbit serum, peptone-yeast extract, heated liver extract, EM defined basal medium, Grace's tissue culture medium, Schneider's Drosophilia medium, blood from *Galleria*, and from the host *Tanytarsus* plus pond water. A combination of pond water, bovine serum, and chick embryo extract (3:1:1) gave the best results.

CONCLUSIONS

From the limited and often incomplete and inconclusive results reported here, it is obvious that development of in vitro methodologies is still at an early stage. However, it should be pointed out that when in vitro culture attempts were first initiated, very little background information on physiological and physical parameters was available on which to base media choice or methodologies for successful culture of mermithids. Indeed, only recently has research yielded data applicable to media formulation for specific nematodes

(7,12). Still, a vast range of physiological criteria pertaining to the host and parasite and their interrelationship has yet to be evaluated in order to solve the many outstanding problems, including the in vitro production of male nematodes. The continuation of such research is essential to expedite in vitro culture. Unfortunately, such programs are generally undersubscribed in money and manpower. Funding should be solicited from governmental, university, and commercial organizations, all of whom have shown increased interest in the development of mermithids as biological control agents. If mass production by in vitro methods is ever to become a reality, it is up to researchers to impress all possible sources of funding with the potential economic benefits inherent in such a system.

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