An Improved Method for Long-Distance Shipping the Mosquito Parasite Romanomermis culicivorax

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Abstract: To prevent losses during long-distance shipment, a new delivery system was developed for the mosquito parasite *Romanomermis culicivorax*. A specially designed shipping container, two types of culture media (sand and a mixture of sand, vermiculite, and charcoal [SVC]), and two types of insert (polystyrene and polyester urethane foam [PUF]) were tested. Cultures shipped in SVC/PUF insert combination produced significantly higher yields of preparasites than did the other three combinations tested, and the sand/polystyrene combination produced significantly lower yields than did either sand or SVC media shipped with the PUF insert. Yields from cultures shipped in sand/PUF and SVC/PUF combinations were significantly lower than from unshipped controls. Maximum losses (52%) occurred in cultures shipped when 13–16-wk old. Also, yields of preparasites were significantly lower from cultures shipped singly when compared to similar cultures shipped in groups of eight. Techniques and procedures developed in this study did not completely solve the loss associated with delivery of *R. culicivorax*. For the first time, however, the system can guarantee the delivery of a quality product. The study defines the importance of the carrier medium, age of cultures, shipment size, and the restriction of the movement of the culture medium.

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The mermithid nematode parasite *Romanomermis culicivorax* Ross and Smith has been extensively studied as a biological control agent of larval mosquitoes. It is environmentally safe for nontarget organisms (1), is readily mass produced (4), and is easily applied to mosquito breeding areas on a large scale to give effective initial control (2) and sometimes long-term control through natural recycling (5). However, the full potential of this parasite has not been realized because successful delivery of viable larvae for extensive field testing in a variety of environmental conditions has not been achieved.

The limited shelf life of larvae and the damage they sustained during shipping and handling were major reasons why one company (Fairfax Biological Laboratory, Inc., Clinton Corners, New York) discontinued attempts at commercial development of *R. culicivorax*.

Losses of *R. culicivorax* caused by extensive shipping and handling have been reported by Petersen and Levy (3). They showed that the shipment of 2–6-wk-old cultures sustained a 38% loss compared to losses in excess of 80% when cultures older than 8 wk were shipped. They concluded that when cultures containing mature eggs are subjected to rough handling, premature hatching occurs and the newly hatched larvae perish. Although transportation of very young cultures (2–4 wk) reduce losses during shipment, the cultures require an additional 5–7 wk after arrival to mature and this often is a major inconvenience to the user. Therefore, it became necessary to develop shipping procedures for mature cultures that provide a reliable product upon delivery. Herein is reported the finding of that research.

MATERIALS AND METHODS

Romanomermis culicivorax used in this study were reared weekly at Nutrilite Products, Inc. (NPI), Lakeview Research Center, Lakeview, California, according to a modification of the procedures of Petersen and Willis (4). Measured quantities of postparasitic nematodes from a given rearing cycle were collected daily, or every other day (depending on the work load), and placed into individual culture (shipping) trays containing the appropriate carrier material until each culture tray had received a total of 5 g of postparasites. This ensures the proper ratio of early emerging males and late emerging females. An average of 80 cultures were established each week.

The culture trays developed and used in this study were designed and constructed based on previous shipping trials involving experimental prototypes made in a variety of sizes and materials. The trays consisted of two parts, each made of 1.47-mm-thick

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vacuum foam molded styrene. The lid (21 × 16 × 1.3 cm) contained tabs on each of the sides to engage a rounded lip protruding outward from the tray (19.5 × 14.5 × 5.5 cm).

Two types of inert carrier material were tested: sand only and a mixture of number 2 blasting sand, industrial grade vermiculite, and charcoal (SVC) at a ratio of 5:1:1. The charcoal was rinsed in cold water for 24 h to remove small particles and then dried prior to use in the carrier mixture. This minimized clouding of the water when preparasites were collected. Each culture tray received ca. 550 cm^3 of sand or SVC material (corresponding to a carrier depth of 1.9 cm), 500 ml of chlorine free water, and 5 g of postparasites. About 400 ml of the water was poured from each culture after 1 wk, and the remainder of the free water was removed with absorbant paper towels after an additional 2 wk. Cultures were then covered with the styrene lid and stored at ambient temperature (24–27 C) until ready for shipment. Cultures were prepared for postal shipment by removing the styrene lid, inserting either a polyester urethane foam (PUF) or an expanded polystyrene (EPS) spacer and replacing the lid to immobilize the carrier material during transit. The PUF spacer (nominal density 2.0 lbs per ft^3) measured 19.5 × 14.5 × 4 cm; the LPS spacer (nominal density 1 lb per ft^3) measured 19 × 14 × 3 cm. The rigidity of the EPS material permitted it to be pressed tightly into the styrene trays thereby immobilizing the carrier material. The PUF insert was partially compressed when inserted between the styrene lid and carrier surface. This compression was found to be necessary to immoblize the carrier material. With the styrene lid securely in place, the cultures were placed in tight, form-fitting EPS shipping containers 2 cm thick on all sides (nominal density 1.5 lbs per ft^3). The EPS shipping container provided some temperature stability and some shock absorbing capacity not provided by the styrene culture tray and lid. All cultures were placed in single walled corrugated shipping cartons (25.4 × 20.5 × 11.7 cm). Cultures selected for bulk shipment, usually in groups of eight cultures, were placed in a larger single-wall corrugated box with EPS packing. Individual or bulk packed cultures were then posted air express to the Gulf Coast Mosquito Research Laboratory, USDA, Lake Charles, Louisiana. Within 1–5 d of their arrival, cultures were flooded for 16 h with 800 ml of well water to induce egg hatch. Cultures ranging in age from 7–20 wk were prepared for shipment. A total of 175 test cultures were posted during the study, 134 bulk shipped and 41 individually shipped. Six 1-cm^3 samples of hatched preparasites from each culture were counted according to procedures described by Petersen and Willis (4).

At the time cultures were prepared for shipment, 67 additional cultures, 38 in SVC medium and 29 in sand, were established from corresponding nematode batches and were retained at the Lakeview facility to serve as unshipped controls. Preparasites were collected from the controls in the same way and on the same day they were collected from the shipped cultures.

RESULTS AND DISCUSSION

There was no significant difference ($P = 0.05$) in yield of preparasites between control (unshipped) cultures established using SVC medium and the sand medium. The SVC medium produced a mean of 2,556 × 10^3 preparasites for all age groups compared with 1,757 × 10^3 for the sand medium. However, significantly greater yields were obtained from 17–18-wk-old cultures than from the other age groups; the 11–16-wk age group produced significantly higher yields of preparasites than the 7–10-wk and 19–20-wk age groups (Table 1).

Cultures were shipped with the SVC or sand media and the EPS or PUF inserts, a total of four shipping combinations. The preparasite yields from these cultures by age at time the cultures were shipped are shown in Table 2. Preparasite yields were significantly higher for cultures shipped in the SVC medium with the PUF insert (average 1,461 × 10^3) than for any of the other three combinations. The combination of sand medium and PUF insert produced the second best yields, but they were not significantly higher than the combination of SVC medium and EPS insert. The sand/EPS combination was significantly lower than

Table 1. Comparison of yields (× 10), by age, of preparasites of *Romanomermis culicivorax* from unshipped and group shipped cultures with the carrier medium immobilized with foam inserts.

Culture age (wk)*	n	Unshipped	n	Shipped	Percent loss of preparasites (gain) from shipment
7– 8	10	648 a†	10	693 z	(6)
9–10	12	1,000 a	12	809 z	19
11–12	11	2,342 b	12	1,745 y	25
13–14	12	2,883 b	11	1,400 yz	51
15–16	12	2,594 b	11	1,232 yz	53
17–18	5	4,664 c	6	2,600 x	44
19–20	5	677 a	9	1,002 yz	(32)
Mean		2,040		1,284	

*At time of shipping.

†Values followed by the same letters within each column do not differ ($P < 0.05$) using Duncan's multiple-range test.

either sand or SVC media shipped with the PUF insert.

When preparasite yields from shipped cultures were compared by age, all except the sand/EPS combination produced highest yields when 17–18 wk old. Also, similar yield patterns were shown for the other age groups for the Sand/PUF, SVC/PUF, and SVC/EPS combinations. However, increase in yields was not linear with age up to the 17–18-wk age group as would be expected. The pattern was similar to that for the unshipped controls. The sand/EPS combination produced uniformly low yields, none of which were significantly different. The highest mean yield for this shipping combination was 815 × 10^3 preparasites and occurred in the 11–12-wk age group.

When the yields of the shipped sand/PUF and SVC/PUF combinations were pooled and compared with the pooled sand and SVC controls (Table 1), all shipped age groups showed substantial reductions in hatch except the 7–8-wk and 19–20-wk age groups. Greatest losses (52%) occurred in cultures shipped when 13–16 wk old. The mean yield from the unshipped group (2,040 × 10^3 preparasites) was significantly higher (< 0.01) than was the mean yield from the shipped group (1,284 × 10^3 pre-

Table 2. Effects of two carrier mediums and two carrier immobilizing inserts* on the yields of preparasites of *Romanomermis culicivorax* (× 10^3) by age when cultures were shipped from California to Louisiana.

Culture age (wk)‡	Sand†				SVC			
	n	Foam insert	n	EPS insert	n	Foam insert	n	EPS insert
7– 8	5	770 ab	5	268 c	5	616 e	5	319 g
9–10	6	636 b	6	417 c	6	982 e	6	461 gh
11–12	6	1,718 a	6	815 c	6	1,771 ef	6	1,315 i
13–14	5	1,009 ab	4	708 c	6	1,726 ef	5	1,208 hi
15–16	5	1,010 ab	5	399 c	6	1,417 ef	6	842 ghi
17–18	1	2,310 a	1	112 c	5	2,658 d	4	1,413 i
19–20	3	772 ab	2	86 c	6	1,117 e	2	150 g
Mean		1,055 x		488 y		1,461 z		861 xy

*The inserts were used to immobilize the carrier during shipment.

†Values followed by the same letters within each column do not differ ($P < 0.05$) using Duncan's multiple-range test.

‡At time of shipping.

Table 3. Mean yields ($\times 10^3$), by age, of preparasites of *Romanomermis culicivorax* cultures maintained in sand:vermiculite:charcoal (5:1:1) carrier medium immobilized with foam inserts and shipped singly or in groups of eight.

Culture age (wk)†	Shipping method*				Percent loss
	n	Groups of 8	n	Singly	
7– 8	5	616 a	8	553 c	10
9–10	6	982 a	8	484 c	51
11–12	6	1,771 ab	8	1,263 d	29
13–14	6	1,726 ab	8	1,041 cd	40
15–16	6	1,417 ab	6	996 cd	30
17–18	5	2,658 b	6	1,067 cd	60
19–20	6	1,117 ab	5	720 cd	46
Mean		1,461 y		872 z	

*Values followed by the same letters within each column do not differ ($P < 0.05$) using Duncan's multiple-range test.

†At time of shipping.

parasites).

When yields of preparasites from comparable cultures shipped singly or in groups of eight were compared, cultures shipped singly averaged 872 × 10 preparasites and those shipped in group packages averaged 1,461 × 10^3. The difference was highly significant (Table 3). Yields from singly shipped cultures 9-wk-old or older were reduced by an average of 40% (29–60%).

The results (Table 1) show that shipping causes substantial reduction in yields of infective stage nematodes. Therefore, it is necessary to select a delivery system that will reduce these losses most effectively. When the two culture media were compared in unshipped cultures, the SVC averaged higher yields of preparasites, though the differences were not significant. However, in shipped cultures with PUF inserts, the SVC medium produced significantly higher yields than those shipped in sand (Table 2). It would appear that the addition of charcoal and vermiculite to the culture sand is of little value for maintaining laboratory cultures, especially when the added time and effort required to incorporate this into the system are considered. However, if *R. culicivorax* cultures are to be shipped, the SVC mixture can apparently reduce yield losses substantially. It is presumed that the water normally expressed from the sand as the cultures are handled during delivery is reabsorbed by the charcoal and/or vermiculite, thereby reducing the chance of free water formation that subsequently acts as a hatching stimulus for the mature nematode eggs.

Immobilization of the culture medium may be the most important factor in reducing preparasite losses. The culture pans were designed to allow for flooding of the cultures to hatch nematode eggs. Thus, this area had to be filled with a spacer to avoid excessive agitation of the culture media. Test results clearly showed that the flexible PUF insert was superior to the rigid EPS insert (Table 2). Observations of cultures as they were received showed little or no shifting of the culture media when PUF was used. However, even when the EPS insert was placed tightly into the cultures, its rigid nature allowed for considerable shifting of the medium during shipment. The combination of PUF insert and SVC media resulted in significantly lower hatching losses than did the other combinations, probably because it prevented damage to the eggs from the shifting sand, increased the absorption of capillary water as it was freed from the carrier material, and provided a less dense medium less likely to crush the eggs. Even with the protections provided by the PUF insert and the SVC carrier, substantial losses still occur (mean of 45% between the ages of 12–18 wk when compared with unshipped controls in the SVC medium). Losses were even greater when the cultures were subjected to the rougher handling presumed to occur when cultures were shipped singly instead of in groups of eight in larger boxes (Table 3).

The mere size of the group shipped cultures was assumed to reduce rough handling.

Age of the cultures was also an important factor in the successful delivery of the nematode product. Petersen and Levy (3) previously demonstrated that losses could be reduced substantially if very young cultures were shipped and allowed to mature at the point of delivery. However, since applicators prefer to disseminate the nematodes soon after delivery, this study only considered the age of the cultures as it affected the yield of preparasites immediately upon delivery to the consumer. Maximum yields were obtained from unshipped cultures hatched when 11–18 wk old. The variation between age group yields reflected in-house variability in rearing system (i.e., numbers used, sex ratios, or health of the nematodes). Similar yield patterns were exhibited for cultures shipped with PUF inserts. However, a different yield pattern was obtained for cultures shipped with the EPS inserts, especially the sand/EPS combination. With this group, the highest preparasite yields were obtained from the 11–12-wk age group with yields decreasing with age thereafter. This again strongly suggests premature hatching or damage to the mature eggs as a result of the shifting sand medium during shipping and handling.

Although the techniques and procedures developed in this study did not eliminate yield loss in shipping *R. culicivorax,* losses were reduced to acceptable levels. A system was developed that for the first time facilitated the delivery of a quality product; the importance of the carrier medium, age of cultures, shipping container size, and the importance of restricting the movement of the culture medium has been described.

The most important problems associated with the preparation and delivery of *R. culicivorax* as a biological control for larval mosquitoes have been solved; the system is ready for commercial exploitation.

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