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Recovery and viability of *Dirofilaria immitis* microfilariae

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Summary

The viability of *Dirofilaria immitis* microfilariae recovered from canine blood by different methods was determined. Microfilaria recovery techniques included saponin lysis, saponin lysis with a trypsin treatment, dextran sedimentation and phytohemagglutinin treatment. Criteria for evaluating viability were microfilarial motility in vitro at 37° C, microfilarial development in mosquitoes and the ability of microfilariae to circulate in mice. Although each method produced motile microfilariae, differences among groups of microfilariae recovered by different techniques were apparent by each of the criteria for viability. Saponin lysis gave superior yields of viable microfilariae.

Key words: *Dirofilaria immitis;* microfilariae; viability.

Introduction

Recovery of large numbers of cell-free, viable microfilariae is requisite to various in vitro and in vivo studies on this parasitic stage. Numerous methods for recovery of *Dirofilaria immitis* microfilariae have been reported (Cherian et al., 1980; Muscoplat et al., 1977; Obeck, 1973; Sawyer and Weinstein, 1963; Wong, 1964); however, when many parasites must be recovered from large quantities of blood these techniques may be inefficient or may yield microfilarial preparations containing cells or insoluble cell debris.

It is often incorrectly assumed that motility of microfilariae is an adequate measure of viability. Although microfilariae that are not motile at 37° C in optimum culture conditions are probably not viable, it cannot be directly inferred that all microfilariae that are motile under those conditions will live in an acceptable host or will undergo development in the mosquito.

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Differences in the infectious potential of blood-derived versus peritoneum-derived microfilariae of *Brugia malayi* have been reported (Schrater et al., 1982) and it has been demonstrated that *Brugia pahangi* microfilariae must mature sufficiently before they will develop to infective larvae (de Hollanda et al., 1982). These observations emphasize the importance of assessing microfilarial viability by criteria other than microscopic observation.

The present investigation was designed to evaluate the viability of *D. immitis* microfilariae recovered from blood by modifications of three previously described techniques which have been used for efficient recovery of microfilariae from large quantities of blood. The criteria for determining viability of isolated microfilariae included: (1) motility of microfilariae in vitro at 37° C, (2) development of microfilariae in the mosquito, and (3) ability of microfilariae to circulate in the mouse.

Materials and Methods

Recovery of microfilariae. Blood from an experimentally infected dog containing approximately 1×10^5 *D. immitis* microfilariae/ml was collected by venipuncture into a heparinized syringe. Microfilariae for each experiment were derived from the same blood sample. Cell-free microfilariae were isolated from blood by four methods. The first method (saponin/trypsin) was a modification of that reported by Sawyer and Weinstein (1963). Heparinized microfilaremic blood was diluted 1:11 in 0.2% saponin with 0.85% NaCl in distilled water. This mixture was incubated at 37° C for 15 min and centrifuged at $900 \times g$ for 5 min. The resulting pellet was washed twice with 0.01 M phosphate buffered saline pH 7.2 (PBS) and resuspended in 10 ml trypsin/saline containing 0.85 g NaCl, 0.02 g $NaHCO_3$, 0.11 g Na_2HPO_4, 0.02 g KH_2PO_4, 0.15 g dextrose and 0.1 g trypsin (1:250) per 100 ml distilled water. The suspension was incubated at 37° C for 15 min and centrifuged as before. The pellet was washed once with PBS, resuspended in 5 ml 0.04% DNase (Sigma DN-100, Sigma Chemical Co., St. Louis, MO, USA) in PBS and gently agitated until a homogenous preparation of microfilariae was observed. DNase was removed by centrifugation, and the pellet was washed once with PBS. The second method (saponin) was identical to the saponin/trypsin method except that the trypsin/saline treatment step was omitted. The third method (PHA) was a modification of a previously described technique (Wong, 1964). Ten ml of heparinized microfilaremic blood was mixed with 0.5 ml phytohemagglutinin P (Difco Laboratories, Detroit, MI, USA) in a 25 ml graduated cylinder and incubated for 45 min at room temperature. The plasma layer was aspirated into a glass tube and placed on ice. The remaining blood suspension was washed in the graduated cylinder with an equal volume of PBS and incubated at room temperature for 30 min. The supernatant was added to the plasma layer; this procedure was repeated for a total of four washes. The combined supernatants were centrifuged as before, and the pellet was gently agitated in 5 ml distilled water for 1 min or until contaminating erythrocytes were lysed. Immediately 10 ml PBS was added to the water suspension and centrifugation, DNase treatment and the PBS wash were repeated as before. The fourth method (dextran) employed was a variation of a dextran sedimentation method for recovering microfilariae (Cherian et al., 1980). Heparinized, microfilaremic blood was mixed 9:1 with 20% dextran (170,000 mol. weight) in a syringe. The syringe was allowed to stand with the needle extended upward for 60 min at 37° C. The needle was then bent at a 45° angle and the dextran layer carefully expressed into a tube. This suspension was centrifuged and washed. Contaminating erythrocytes were lysed with distilled water. The pellet was treated with DNase and washed once with PBS.

In vitro culture. Seven thousand microfilariae from each of the separation methods were inoculated into flat-sided tubes containing 2 ml F12(K) with 5% heat inactivated fetal calf serum and

gentamicin (100 μg/ml) and incubated at 37° C. Media were changed and microfilariae were examined microscopically every 48 hours for up to 5 weeks. Cultures were monitored for microfilarial motility; the number of motile parasites was determined and expressed as a percentage of the total number of parasites counted in three to six randomly selected microscopic fields.

Mosquito inoculations. Aedes aegypti were of the Liverpool strain originally selected for susceptibility to *Brugia malayi* (Macdonald, 1962). This strain is also highly susceptible to infection with *D. immitis* (Macdonald and Ramachandran, 1965). Larval mosquitoes were reared at 28° C on a diet which consistently gave 80–90% pupation by day six after hatching. Adults emerged synchronously on days eight and nine after hatching and were maintained at 28° C and 80% RH with 15 hours of light per day.

Microfilariae of *D. immitis* isolated by the four test procedures were inoculated into female *Aedes aegypti* using the fluid enema technique described by Klowden (1981) for *Brugia pahangi.* A dose of 30 microfilariae was administered to each female. Inoculated mosquitoes were maintained in darkness at 28° C and 80% RH and were fed 5% sucrose. On days 14–16 after inoculation the mosquitoes were anesthetized and dissected thoroughly in Hayes' (1953) saline to determine the extent of parasite development. Numbers and developmental stages of larval *D. immitis* recovered from each mosquito were recorded. Third-stage larvae (L_3s) were recognized by the presence of a patent rectum and an esophagus clearly differentiated into muscular and glandular segments. Mean length and width measurements ($\pm$ SD) for L_3s fixed in Bles fluid (Wharton, 1959) were 986 $\pm$ 92 μm and 25 $\pm$ 1 μm, respectively (N = 8). The mean number of L_3s per mosquito was calculated for each treatment group and was used as a basis for comparing the microfilarial isolation procedures.

Mouse inoculations. Female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA, USA) aged 6–10 weeks were irradiated and inoculated with microfilariae as described previously (Grieve and Lauria, 1983). Sixteen, 23, 30 and 37 days after inoculation, blood was collected and microfilariae were counted (Grieve and Lauria, 1983). In each of three separate experiments microfilarial counts of 7 mice inoculated with microfilariae recovered with either saponin/trypsin, PHA or dextran were compared to counts from 6 mice inoculated with microfilariae prepared by saponin. Cohorts in separate experiments were bled at the same time of day, usually 11.00–14.00 h.

Statistics. Chi-square or Students' t test were used to determine if there were significant ($p<0.05$) differences between groups in the in vitro culture and mosquito inoculation experiments. An analysis of variance was used to compare multiple means. In the mouse inoculation experiment significant differences between the groups and over time were determined using a BMDP statistical software package for analysis of variance and covariance, with repeated measures (Dixon, 1981) on a DEC 10/20 computer.

Results

Microfilariae recovered by each method and maintained in vitro at 37° C were motile for up to 5 weeks. However, when the percentage of motile microfilariae in each treatment group was calculated differences were apparent (Table 1). Differences among groups in the first experiment were significant. Saponin/trypsin adversely affected the motility of microfilariae in the first and second experiments. Differences in motility of microfilariae recovered by saponin and dextran were not significant and, although differences in motility of microfilariae recovered by saponin and PHA were significant, these differences should be interpreted cautiously because multiple comparisons were made between groups.

Table 1. Motility of *D. immitis* microfilariae isolated by four different methods and maintained in vitro at 37° C

Experiment	Days post inoculation	Isolation method	Number of microfilariae counted	Number (percent) of motile microfilariae	
1	12	saponin	79	66 (83.5)[a]	$p < 0.0001$
		saponin/trypsin	71	18 (25.4)[b]	
		dextran	74	56 (75.7)[c]	
		PHA	76	52 (68.4)[d]	
2	10	saponin	150	127 (84.7)	$p < 0.0001$
		saponin/trypsin	129	84 (65.1)	

[a–b] $p < 0.0001$
[a–c] not significant
[a–d] $p < 0.05$

Table 2. Effect of microfilariae isolation method on the in vivo development of *D. immitis* microfilariae to third-stage larvae

Isolation method	Mosquitoes dissected	Mosquitoes infected	Larvae/mosquito (±SE)*	
Saponin	15	15	7.6 (±1.12)[a]	$p < 0.001$
Saponin/trypsin	10	2	0.2 (±0.13)[b]	
Dextran	10	10	6.5 (±0.76)[c]	
PHA	12	7	2.3 (±0.76)[d]	

* Original inoculation of 30 microfilariae/mosquito
[a–c] not significant
[a–d] $p < 0.0001$
[c–d] $p < 0.002$

Microfilariae recovered by different methods differed significantly in ability to develop to infective larvae within mosquitoes (Table 2). No significant differences were found between saponin and dextran; however, the number of L_3s recovered from mosquitoes inoculated with PHA-derived microfilariae was significantly less than the number of L_3s developing from microfilariae recovered by saponin or dextran. Saponin/trypsin-derived microfilariae demonstrated the poorest capability for developing to L_3s.

Microfilariae recovered by saponin/trypsin, PHA and dextran did not circulate in mice to the extent of microfilariae recovered by saponin (Fig. 1). Mean microfilaria numbers differed significantly between saponin and saponin/trypsin ($p < 0.0003$), saponin and PHA ($p < 0.0001$) and saponin and dextran ($p < 0.02$). The change in microfilaria numbers over time was significantly different between saponin and saponin/trypsin ($p < 0.02$), saponin and PHA ($p < 0.04$), but not different between saponin and dextran.

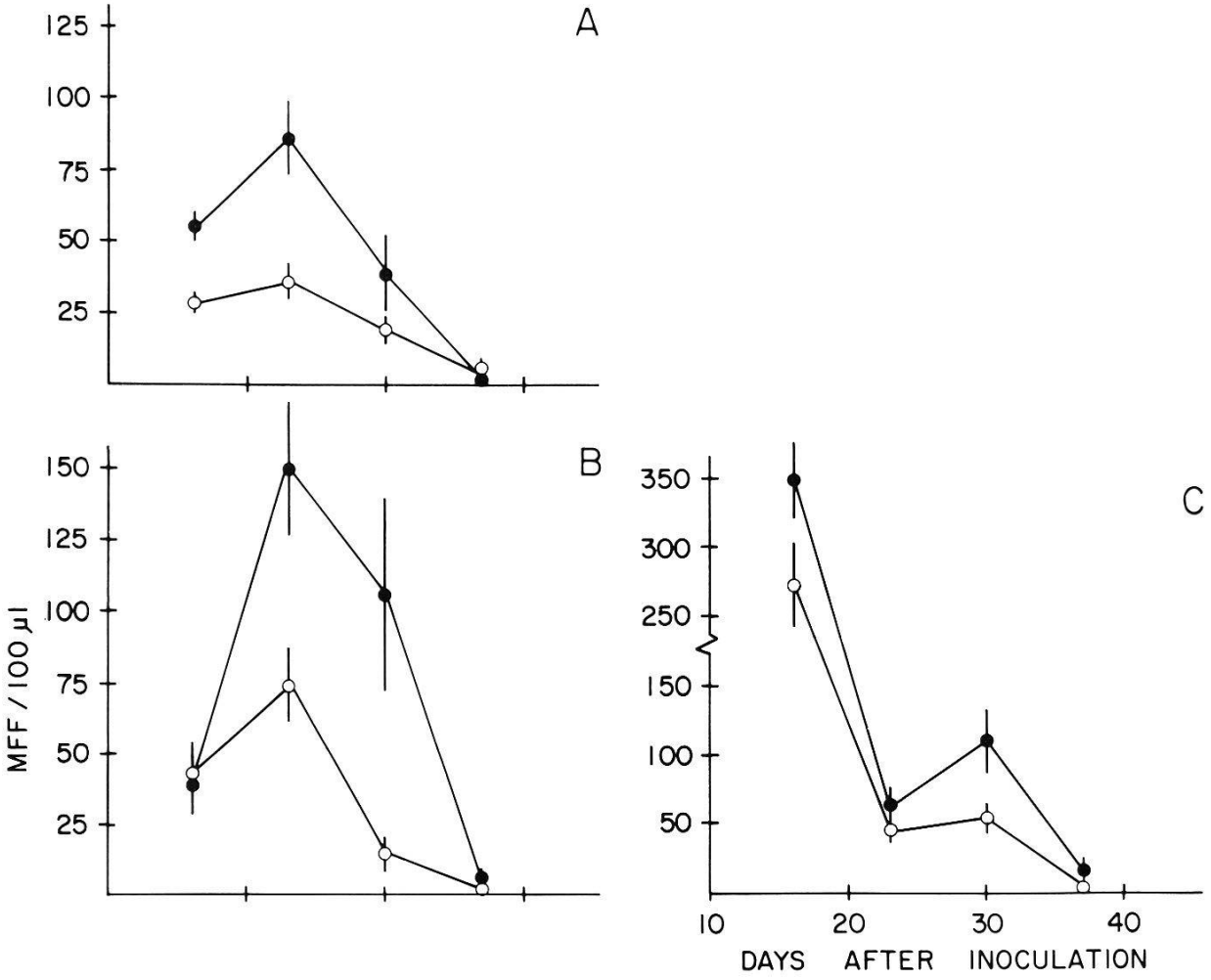

Fig. 1. *Dirofilaria immitis* microfilaria numbers in BALB/c mice inoculated with microfilariae recovered by different methods. A. ●——● saponin, ○——○ saponin/trypsin. B. ●——● saponin, ○——○ PHA. C. ●——● saponin, ○——○ dextran. Each point for each saponin group represents the mean of six mice. Points for all other treatment groups represent the mean of seven mice. Vertical bars represent standard errors of the means.

Discussion

Although microfilariae recovered by each method and maintained in vitro at 37° C were motile for up to 5 weeks, differences in the percent of motile microfilariae existed among treatment groups. Saponin and dextran yielded the most motile microfilariae and saponin/trypsin had an adverse effect on motility.

Data on recovery of L_3s from cohorts of mosquitoes inoculated by fluid enema indicate that saponin yields the highest proportion of infective microfilariae. Infectivity of microfilariae isolated by dextran approached that of saponin-recovered parasites while microfilariae isolated by PHA and saponin/trypsin exhibited relatively low infection potential.

The enema technique is a useful tool in physiological, behavioral and parasitological studies of mosquitoes (Briegel and Lea, 1975; Klowden and Lea, 1975a; Klowden, 1981) and black flies (Klowden and Lea, 1975b). It allows the

investigator to administer known numbers of microfilariae to a susceptible arthropod and quantitatively evaluate their ability to escape the gut, invade the target tissue and develop to L_3s. Quantitative control of developmental parameters such as these may be particularly important in in vitro studies which necessitate subjecting microfilariae to potentially stressful treatment such as isolation from whole blood or skin, cryopreservation (Schiller et al., 1979) and artificial exsheathment (Devaney and Howells, 1979a, 1979b) prior to inoculation into the experimental system. Recent work has demonstrated that the majority of *B. malayi* microfilariae from the peritoneal cavities of jirds fail to penetrate the midgut of susceptible *Ae. aegypti* (Schrater et al., 1982) and that *B. pahangi* microfilariae must mature before they will develop to L_3s (de Hollanda et al., 1982). These findings emphasize the need for a quantitative control for microfilarial infectivity. Use of the enema technique in preference to intrathoracic injection is crucial in the case of *D. immitis* since microfilariae of this species do not penetrate the midgut wall and enter the hemocoel, but rather migrate directly from the gut lumen to the malpighian tubules where larval development occurs.

The ability of *Onchocerca gutturosa* and *O. gibsoni* microfilariae to migrate in mice has been used as a criterion for viability following purification (Forsyth et al., 1982) and cryopreservation (Ham et al., 1979) of microfilariae. Microfilariae were recovered from euthymic and athymic mice up to 6 days and 20 days, respectively. Sublethally irradiated mice were used in the present investigation because previous work demonstrated that microfilaremia could be extended from 3 weeks in nonirradiated mice to 6 weeks in irradiated mice (Grieve and Lauria, 1983). Extending the microfilaremia permitted measurement of both the differences in total microfilaria numbers between groups and also differences between groups in the change of microfilaria numbers over time.

Since saponin-recovered microfilariae appeared to be the most viable by the other criteria in this study and by preliminary experimentation in mice and, because of technical limitations, each of the three other recovery techniques were compared to saponin in individual experiments. Microfilariae recovered by saponin/trypsin, PHA and dextran did not circulate to the extent of microfilariae recovered by saponin. In addition to differences in total numbers, the decrease in microfilaria numbers over time was significantly greater in mice inoculated with microfilariae isolated by saponin/trypsin and PHA. Differences in the total number of microfilariae between groups suggests that saponin/trypsin, PHA and dextran adversely affected a portion of microfilariae which were killed or would not circulate upon inoculation into mice. Differences in microfilaria numbers over time in mice inoculated with saponin/trypsin- and PHA-derived microfilariae indicate that another population of microfilariae was impaired by those treatments and was either killed more rapidly, or more rapidly lost the ability to circulate, than saponin-derived microfilariae.

Since microfilarial periodicity has been reported in mice inoculated with *B. malayi* (Grove et al., 1979), cohorts of mice in the present study were bled at the same time of day. Although an effort was made to bleed mice at the same time of day among the experiments, at day 17 in the dextran experiment mice were bled at 23.00 h, approximately 12 hours later than usual. The difference in collection time accounts for the abnormally high numbers of microfilariae on that day in that experiment. This is consistent with subsequent research which demonstrated that *D. immitis* microfilaremia in the BALB/c mouse is subperiodic and nocturnal (Grieve and Lauria, 1983). Higher microfilaria numbers at day 17 changed the appearance of the course of microfilaremia in that experiment. Although total microfilaria numbers were different between groups, the change in microfilaremia over time was not different. If mice were bled at the usual time of day on day 17, it is possible that a difference in microfilaremia would have been observed in mice inoculated with dextran-derived microfilariae.

The isolation techniques tested gave varying recoveries of viable microfilariae. Saponin appeared to be the best of four different microfilaria recovery techniques evaluated in this study. It yielded large numbers of cell-free microfilariae and was superior in viability overall based upon three methods of evaluation.

Determination of an acceptable microfilaria recovery technique was an important goal of this study. Equally important conclusions from the data are that routine manipulation of microfilariae may be harmful and that valid quantitative viability assays should be incorporated into any study concerned with experimental treatments on microfilariae.

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