DOSE—RESPONSE STUDIES OF A NEW SPECIES OF *PER OS* AND VERTICALLY TRANSMITTABLE MICROSPORIDIAN PATHOGEN OF *AEDES AEGYPTI* FROM THAILAND[1]

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ABSTRACT. Results are presented of dose-response studies of a new species of *per os* and vertically transmittable microsporidian pathogen of *Aedes aegypti* from Thailand. The relationship between duration of exposure and percent transmission to *Ae. aegypti* larvae was determined. Estimated IC_{50}s for 1st, 2nd, 3rd and 4th stage *Ae. aegypti* larvae were 54, 110, 210 and 1100 spores/ml, respectively. Concentrations of about 10^4, 5×10^4, 10^5 and 5×10^5 spores/ml for 24 hr were required to cause cumulative mortality of 90% or more by the time the exposed population was 14 days of age (post-hatch), when exposures were made to 1st, 2nd, 3rd and 4th stage larvae, respectively. The low IC_{50}s indicate the pathogen is very infectious, while the LC_{90}s suggest it is not very virulent. The fact that it might be dispersed by survivors within the target population by vertical transmission enhances its attractiveness as a potential microbial control agent. Infectivity to *Ae. taeniorhynchus* was demonstrated. *Culex quinquefasciatus* and *Anopheles stephensi* were not susceptible.

INTRODUCTION

The hypothetical microbial control potential of microsporidian pathogens for mosquitoes is based on their prevalence and wide distribution in mosquito populations (Hazard and Chapman 1977) and on their demonstrated pathogenicity in laboratory studies (Savage and Lowe 1970, Reynolds 1970, Anthony et al. 1972, 1978a; Undeen and Alger 1975, Anthony and Kelly 1979, Haq et al. 1981, Kelly et al. 1981). Cost-effective larvicidal action has not been demonstrated for these agents in comparison to chemical insecticides, but few field studies of the microbial control potential of microsporidia for mosquitoes have been conducted (Reynolds 1972, Anthony et al. 1978b).

Assessment of the possible usefulness of microsporidia for mosquito control necessarily begins with laboratory studies. These have been inhibited because only 3 known species of mosquito microsporidia can be transmitted at will in the laboratory by *per os* exposures. *Nosema algerae* Vaura and Undeen and *Vavraia culicis* (Weiser) are readily transmittable, but *Microsporidium milleri* (Hazard and Fukuda) sometimes gives unpredictable results (Hazard and Fukuda 1974, Hembree 1979).

A new species of microsporidia that was both vertically transmittable and transmittable by *per os* exposure of host larvae was recently reported infecting the fat body of *Aedes aegypti* (Linn.) from Thailand (Hembree 1979) and has been under preliminary evaluation as a microbial control agent in this Laboratory. According to E. I. Hazard (personal communication), it is a new species with a unique developmental cycle. As presently known, it does not appear to fit conveniently into any of the taxonomic categories below Order Microsporida in the system proposed by Sprague (1977). Since delays are anticipated in naming the species and placing it taxonomically, the results of dose-infectivity and dose-

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mortality studies will be provided at this time.

METHODS

The microsporidian was established in laboratory culture, using a Thai strain of *Ae. aegypti* as host, at the Department of Medical Entomology, US Army Medical Component, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. The pathogen was transported to the United States in infected *Ae. aegypti* eggs and initially continued in culture in the Thai strain of the host at the US Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, MD. Infectivity experiments indicated no difference in the infectivity of the agent to a US strain of the host, so all further studies were conducted with the local strain.

The local host strain has been maintained for several years at this Laboratory. Larvae were reared at 26–27° C and fed a 1:1:1 mixture of yeast, liver powder and ground high protein content hog chow. First pupation occurred during the fifth day in uninfected material and was mostly completed within 3 days, indicating good rearing conditions. Adults were held at 25° C and 80% RH. Eggs were collected on wet paper toweling and held at room temperature. For experiments, eggs no more than 1 month old were used.

The pathogen produced 2 kinds of spores, only one of which caused infections under our conditions. The uninfectious spore (T_1) was produced first, in the *per os* exposed generation, but in only small numbers. A few infectious spores (T_2) were produced in small numbers of *per os* exposed larvae whose retarded larval development continued past normal pupation time. Large numbers of T_2 spores, up to 9×10^5/larva, were produced in a portion of the progeny of the *per os* exposed generation (the F_1 generation) and appeared within 5 days of hatch. These spores were used exclusively to propagate the pathogen in the laboratory and in experiments. A few T_1 spores were produced in F_1 larvae with retarded development that survived past normal pupation time. It was possible to get suspensions of only one kind of spore by harvesting T_1 spores early in the exposed generation and T_2 spores early in the F_1 generation.

To make exposures both for propagating the pathogen and for experiments, infected larvae were triturated in Ten Broeck type tissue grinders, and the spores were counted with a hemocytometer. To provide larvae of relatively homogeneous age for use in experiments, eggs were force hatched by placing them in deoxygenated water containing food for 30 min, followed by 30 min under negative pressure of about 17 psi. Fifty ml glass beakers were used as exposure containers. One hundred larvae of the desired age were placed in these in 19 ml H_20, and spores were added in 1 ml to bring the total volume to 20 ml. A small amount of food was added. For spore production, 24 hr old larvae were exposed to 1000 spores/ml for 24 hr. Exposures were terminated by pouring the contents of the exposure container onto an organdy cloth screen and gently rinsing the larvae with tap water. Exposed larvae were reared in enameled pans with 1 liter tap water, 100 larvae per pan. Infected adults were reared and ova collected as above. Infected 4th stage F_1 larvae were easily identified by placing them in black pans under a bright light. Subcuticular cysts of spores were visible as white spots on the thorax and on all abdominal segments.

Percent infection was determined by examining at least 25 larvae from exposed groups. These were prepared by using the tips of wooden applicator sticks to smear them on glass microscope slides, five per slide, fixing the smears in absolute methanol and staining them for 1 hr in 2% Giemsa stain in 0.01 M phosphate buffer, pH 7.2. Coverslips were affixed with synthetic mounting medium. Smears were examined at 650X magnification. A sufficient number of developing stages of

the pathogen were present 4 days after exposures (or post-hatch, in the F_1 generation) for infected specimens to be identified by a single pass across 1 cm smears reading adjacent microscope fields. Infections as young as a few hours could be detected by reading the whole smear. Many older infections could be identified in a single field.

In all dose-infectivity experiments controls were reared in parallel to exposed material from the same hatch and examined to confirm the absence of pre-exposure infections. To determine the relationship between duration of exposure to the microsporidian and percent transmission, groups of 100 24 hr old *Ae. aegypti* larvae were exposed for 2, 4, 8, 16 and 24 hr to concentrations of 100, 500, 10^3 and 5×10^3 spores per ml.

To determine the relationship between age of larvae and their susceptibility to the microsporidian, 4 age groups were exposed for 24 hr to 5 replicates of various concentrations of the pathogen. Larvae 2 hr old (1st stage) were exposed to concentrations ranging from 25 to 500 spores/ml. Larvae 24 hr old (2nd stage) were exposed to concentrations ranging from 50 to 10^3 spores/ml. Larvae 48 hr old (3rd stage) were exposed to concentrations ranging from 100 to 5×10^3 spores/ml. Larvae 72 hr old (4th stage) were exposed to concentrations ranging from 500 to 10^4 spores/ml. The percents of transmission acquired for the 5 replicates of each age/dose group were averaged. For each age group these were plotted on log-probit paper, regression lines were eye-fitted and IC_{50}s for this pathogen in each instar of the host were estimated.

To determine the dose-mortality relationship between this pathogen and *Ae. aegypti*, larvae 2, 24, 48 and 72 hr old were exposed for 24 hr to various concentrations of spores. These ages corresponded approximately to the 1st through the 4th larval stages, respectively. The concentrations the various larval stages were exposed to were: first stage, 50- 5×10^4 spores/ml; second stage, 50- 10^5 spores/ml; third stage, 100- 5×10^5 spores/ml and fourth stage, 500- 5×10^5 spores/ml. Five groups of 100 larvae were exposed in each age/dose group. Live pupae were collected and placed in cages, one cage for each age/dose group. Daily, beginning at termination of exposure, all dead larvae, pupae and adults were collected and counted. Daily mortality was tabulated. Five groups of 100 unexposed larvae, handled and reared exactly like the treated groups, but not exposed to spores, provided control mortality data for each age group. The experiment continued for 28 days after the initiation of exposure. The entire experiment was conducted twice. Daily mortality counts for the 2 experiments were averaged and used to form one cumulative mortality curve for each age/dose groups and the control groups.

To determine if this pathogen was transmittable to other mosquito species, 1st stage *Aedes taeniorhynchus* (Wied.) larvae, 2nd stage *Culex quinquefasciatus* Say larvae, and 3rd stage *Anopheles stephensi* Liston larvae were exposed for 24 hr to spore suspensions using the same methods described above.

RESULTS AND DISCUSSION

Repeated examination of control larvae confirmed the absence of pre-exposure infections. As shown in Table 1, exposures as brief as 2 hr at concentrations as low as 100 spores/ml resulted in successful transmission. At low concentrations there appeared to be a slight ad-

Table 1. Percent transmission of a new microsporidian to 24 hr old *Aedes aegypti* larvae resulting from various durations of exposure.

Concentration (Spores/ml)	Durations of exposure (hr)				
	2	4	8	16	24
100	44	50	50	53	58
500	72	76	80	84	92
1,000	100	100	100	100	100
5,000	100	100	100	100	100

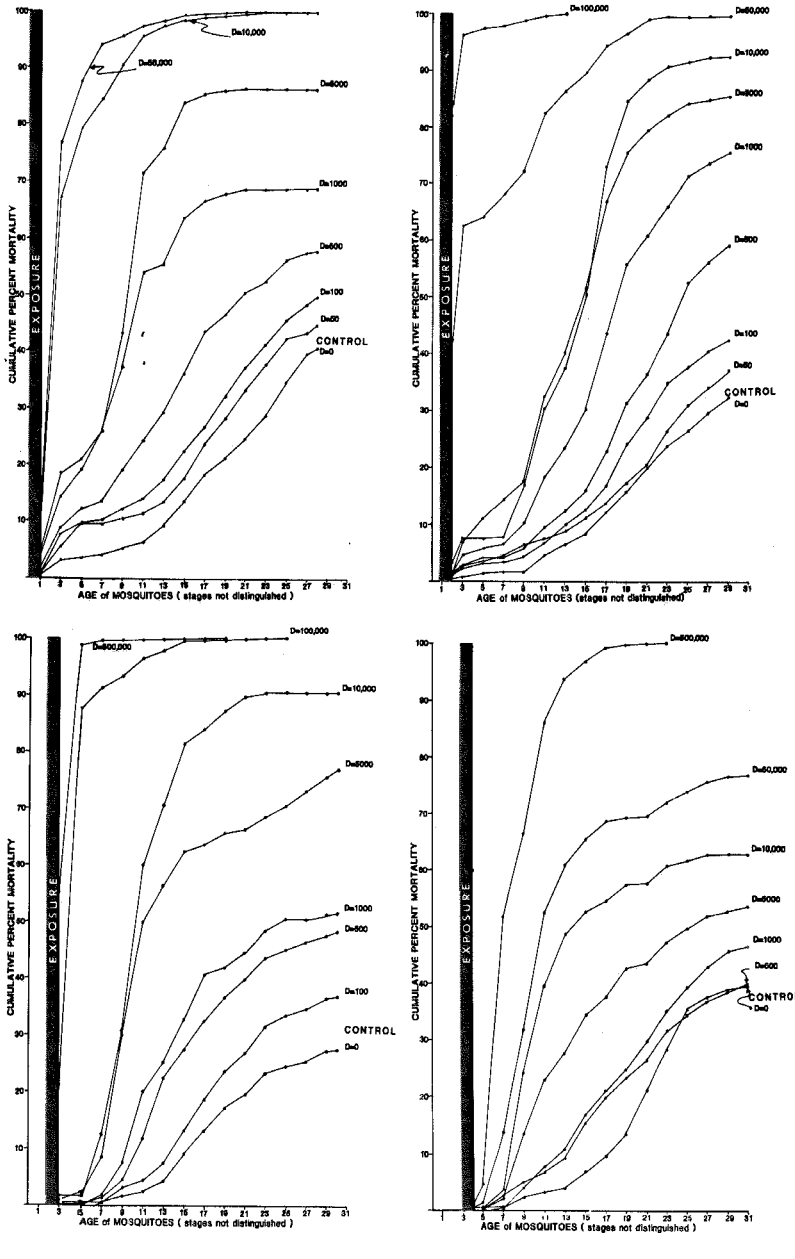

Fig. 1. Cumulative dose-mortality curves for a new microsporidian in *Aedes aegypti* larvae exposed for 24 hr during each larval stage. D = concentration in spores/ml.

vantage to extending exposures up to 24 hr. At the concentration used in our routine pathogen production, 10^3 spores/ml, 100% infection resulted from only 2 hr exposures.

Susceptibility of larvae to *per os* exposures decreases with increasing age as shown in Table 2, but 100% infection rates could be achieved in even 4th stage larvae at modest doses. Estimated IC_{50}s increased from 54 spores/ml for 1st stage larvae to 1100 spores/ml for 4th stage larvae. Concentrations required to reliably give 100% infections under these conditions increased from 500 spores/ml for 1st stage larvae to 10^4 spores/ml for 4th stage larvae. The standard deviations reflect the variability expected when two variable living systems are combined and manipulated under imperfect conditions.

As shown in Fig. 1, very high concentrations were required to effect high mortalities quickly. If a population reduction of 90% by age 14 days post-hatch were arbitrarily selected as necessary to prevent transmission of a vector-borne disease, this could hypothetically be achieved, if larval populations in the 1st, 2nd, 3rd and 4th stages were treated to concentrations of 10^4, 5×10^4, 10^5, and 5×10^5 spores/ml, respectively. Considering that microsporidia can presently be produced in large quantities only in living hosts and that only 9×10^5 spores/larvae have been recovered from *Ae. aegypti* larvae, producing sufficient material to treat many *Ae. aegypti* breeding sites, with the objective of achieving control such as could be achieved by a chemical insecticide, would appear to be a prohibitively

Table 2. Percent transmission resulting from exposure of *Aedes aegypti* of various ages to various concentrations of spores of a new microsporidian.

Concentration (Spores/ml)	Replicate 1	2	3	4	5	Mean	s.d.
	2 hr old (estimated IC_{50} = 54 spores/ml)						
25	24	24	32	36	28	28.8	5.2
50	36	28	44	40	52	40	8.9
100	72	80	88	76	80	79.2	5.9
250	92	96	100	100	100	97.6	3.6
500	100	100	100	100	100	100	0
	24 hr old (estimated IC_{50} = 100 spores/ml)						
50	20	16	20	36	16	21.6	8.3
100	52	52	56	56	60	55.2	3.3
250	52	56	88	68	84	69.6	16.1
500	88	96	96	100	96	95.2	4.4
1,000	100	100	100	100	100	100	0
	48 hr old (estimated IC_{50} = 210 spores/ml)						
100	24	8	20	16	16	16.8	5.9
500	92	88	92	88	80	88.0	4.9
1,000	96	100	88	100	100	96.8	5.2
2,500	100	100	100	100	100	100	0
5,000	100	100	100	100	100	100	0
	72 hr old (estimated IC_{50} = 1100 spores/ml)						
500	28	20	16	20	20	20.8	4.4
1,000	20	20	36	40	24	28.0	9.4
2,500	60	72	80	80	72	72.8	8.2
5,000	84	100	96	96	100	95.2	6.6
10,000	100	100	100	100	100	100	0

difficult task. Therefore, as advised by Anthony et al. (1978a) for *Nosema algerae*, any evaluation of the microbial control potential of this agent should concentrate on the debilitative effects of sublethal infections such as reduced fecundity, life span and flight range and possibly altered behavior. The fact that it is vertically transmittable, and thus dispersable within the host population, must be appreciated.

As shown in Table 3, the microsporidian is almost as infectious to *Ae. taeniorhynchus* as to *Ae. aegypti*, presenting the possibility of usefulness against other mosquito species of medical or economic importance. The estimated IC_{50} for 24 hr old *Ae. taeniorhynchus* was 250 spores/ml. However, we were unable to transmit the pathogen to 2nd stage *Cx. quinquefasciatus* at concentrations as high as 10^6 spores/ml and to 3rd stage *An. stephensi* at a dose of 10^5 spores/ml.

Table 3. Percent transmission of a new microsporidian to 24 hr old *Aedes taeniorhynchus* larvae after 24 hr exposure to various concentrations.

Concentration (Spores/ml)	Test number					Mean	s.d.
	1	2	3	4	5		
100	16	38	38	8	52	30.4	18
500	52	80	80	48	76	67.2	15.8
1,000	56	90	90	60	92	77.6	18
5,000	100	100	100	92	100	98.4	3.6

Direct comparison of potential efficacy between this pathogen, *V. culicis* and *N. algerae* are not possible because methods of evaluation are not standarized. However, they seem to have in common high infectivity, for some host species at least, but low virulence. High transmission rates with *M. milleri* have not yet been reliably achieved, making it presently the least prospective microbial control agent among the microsporidia of mosquitoes that can be transmitted by *per os* exposures. For a full assessment of the microbial control potential of the pathogen studied here, many characteristics remain to be evaluated, especially safety, mass production potential, storage properties, resistance to environmental factors that might degrade its infectivity, field efficacy, host spectrum and nonlethal effects.

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LINKAGE STUDIES ON A NEW MUTANT, BLEACHED PUPA, IN *AEDES (FINLAYA) TOGOI*

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ABSTRACT. A recessive mutant, bleached *pupa (bl)*, was isolated from the Taipei (Taiwan) strain of *Aedes (Finlaya) togoi*. The *bl* pupae are yellowish but brighter than the *y* (yellow larva) pupae. Although the 2 phenotypes cannot be readily distinguished in their pupal stage, they are not identical nor allelic. The *bl* allele is linked to *c*(curved wing) and *pm* (plum eye) in linkage group III. The *bl-c* map distance is 14.2 map units in females and 17.8 units in males. The gene sequence in linkage group III appears to be: *pm* - (40 units) *-y-* ? *-bl-* (14-18)- *c*.

In the review of genetics of *Aedes (Stegomyia) aegypti* (Linn.) made by Craig and Hickey (1967), all 3 linkage groups of this mosquito had been marked with 28 mutant loci, and a total length of these linkage groups was 110 map units. Twelve years later, a new linkage map for this species was reconstructed with a total of 60 marker loci including 14 enzyme markers; the total map length was 156 units (Munstermann and Craig 1979).

About 15 genetic markers have been placed in all 3 linkage groups of *Aedes (Finlaya) togoi* (Theobald), a vector of filarial worms in Asia (Tadano 1980). The map lengths of linkage groups I, II and III are approximately 60, 80 and 60 units, respectively; thus far a total length in this species has become about 200 map units. Therefore, it can be speculated that the total map length in *Ae. togoi* may be longer than that in *Ae. aegypti*.

A new recessive body color mutant, bleached pupa (*bl*), was recovered from an inbred line of the Taipei (Taiwan) strain. A similar mutant named 'bleached' (*b*) has been described in *Culex pipiens* Linn. by McClelland (1978), but the *bl* mutant is not homologous to the *b* mutant in that *b* affects all developmental stages while *bl* only affects the larval and pupal color.

This paper presents results of linkage studies on the *bl* mutant.