EVALUATION OF THE MICROBIAL CONTROL POTENTIAL OF A *HELICOSPORIDIUM* SP. (PROTOZOA:HELICOSPORIDA) FROM *AEDES AEGYPTI* AND *CULEX QUINQUEFASCIATUS* FROM THAILAND[1]

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ABSTRACT. A preliminary assessment was made of the microbial control potential of a *Helicosporidium* sp. (Protozoa:Helicosporida) from *Aedes aegypti* and *Culex quinquefasciatus* in Thailand. *Aedes aegypti* was used as an experimental host in which to determine dose-mortality efficiency of the pathogen. Its storage properties and its susceptibility to degradation by various stressors likely to be encountered in the aquatic environment, such as heat, pH, salinity, detergents, and ultraviolet light were analyzed. The infectivity of the helicosporidian spore to the fall armyworm, *Spodoptera frugiperda,* by injection and reduced infectivity of the resulting spores was demonstrated. The infectivity of the pathogen for *Anopheles dirus, An. maculatus, Ae. taeniorhynchus,* and *Toxorhynchites splendens* was quantified. It was concluded that the agent was unlikely to be useful as a practical, cost-effective microbial control agent until various technological advances are made.

INTRODUCTION

The development of microbial control agents as components of integrated pest management programs for mosquitoes is desirable because of the importance of our aquatic ecosystems and because of the sensitivity of these ecosystems to chemical control agents. Several hundred microbial pathogens of mosquitoes are known (Roberts and Strand 1977, Roberts and Castillo 1980), but the microbial control potential of only a few has been investigated. Because of funding limitations and the small number of investigators engaged in this area of research, it is highly desirable that prospective microbial control agents receive a preliminary screening to assess their control potential, so that scarce resources for further development will be expended only on agents with good probability of being useful. Those selected for definitive evaluation and development should be competitively chosen from among others that have been evaluated in a consistent and programmatical way. Unanimity among investigators as to how pathogens should be screened is not expected. However, a prospective microbial control agent will have to meet certain criteria for its development to be economically feasible. These criteria are: (1) agents must be safe to man and to other important nontarget species; (2) they must be effective in reducing the target population to the desired level within whatever time constraints exist; (3) they must be economically producible in sufficient quantity to satisfy demand; (4) they must have an economically suitable shelf life and (5) they must resist degradation in the environment for a sufficient time to make the necessary contact with the target host. Screening procedures should be oriented to these practical considerations. Figure 1 is a flow chart of events applicable to the preliminary evaluation of microbial control potential.

This paper is a report of efforts conducted within this framework, with the objective of a preliminary assessment of the microbial control potential of a *Helicosporidium* sp. (Protozoa:Helico-

[1] The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. Use of proprietary names does not constitute indorsement.

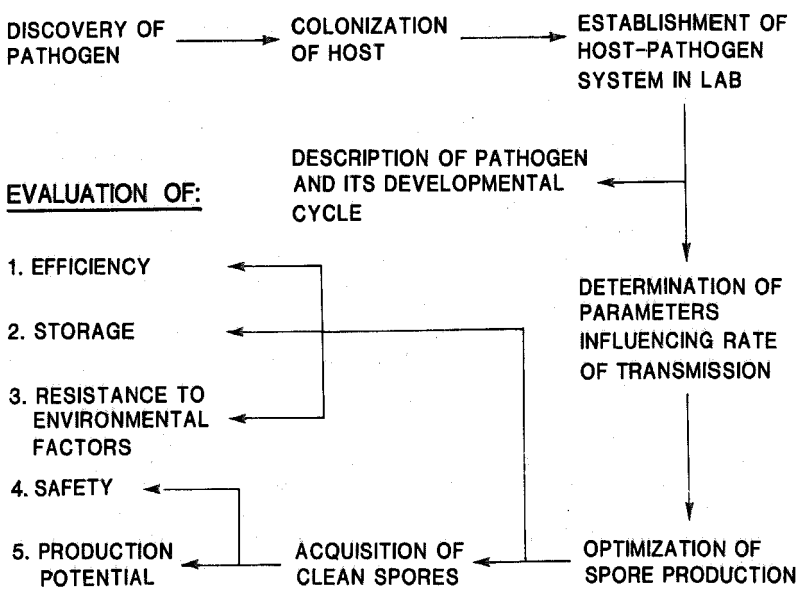

Fig. 1. Critical path of events from the discovery of a mosquito pathogen to the preliminary evaluation of its microbial control potential.

sporida) found in *Aedes aegypti* (Linn.) and *Culex quinquefasciatus* Say in Thailand (Hembree 1979). Safety evaluation was beyond the staff resources of this laboratory. However, before taking the agent out of Thailand, 4×10^7 spores were intubated into the stomachs of each of 12 golden hamsters. After 30 days, these were sacrificed, dissected and examined by a veterinarian for gross pathology, with negative results. The remaining aspects of preliminary evaluation are addressed to varying extents below.

The Helicosporida are little known arthropod pathogens with remarkable host and geographical range, having been found in 3 classes of Arthropoda and on 4 continents (Keilin 1921, Chapman et al. 1967, Weiser 1970, Kellen and Lindegren 1973, Chapman 1974, Fukuda et al. 1976, Sayre and Clark 1978, Hembree 1979). Only 1 species has been named, *Helicosporidium parasiticum* Keilin, 1921. Developmental cycles have been proposed by Keilin (1921) and by Kellen and Lindegren (1974). The ultrastructure of developing stages and spores was provided by Lindegren and Hoffman (1976), who demonstrated that the Helicosporida were Protozoa, not primitive fungi, as once proposed (Weiser 1964). Their relationship to other Protozoa is uncertain.

Naturally occurring infections of Helicosporida in mosquitoes have been found in *Cx. territans* Walker by Chapman et al. (1967) and *Cx. nigripalpus* Theobald by Fukuda et al. (1976) in Louisiana. By exposures in the laboratory, *Cx. territans* could be infected with a helicosporidian from *Carpophilus mutilatus* (Coleoptera: Nitidulidae) (Chapman 1974) as could *Cx. pipiens quinquefasciatus* (=*Cx. quinquefasciatus*) (Kellen and Lindegren 1973). However, Fukuda et al. (1976) were unable to infect *Cx. quinquefasciatus* with the helicosporidian from *Cx. nigripalpus*, suggesting that the helicosporidia from *Carpophilus mutilatus* and *Cx. nigripalpus* were different forms. *Aedes aegypti* was susceptible by laboratory exposure to both the

helicosporidian of *Cx. nigripalpus* (Fukuda et al. 1976) and a form occurring in laboratory cultures of the cladoceran, *Daphnia magna* (Sayre and Clark 1978). Helicosporidia were also found in *Ae. aegypti* and *Cx. quinquefasciatus* from Thailand (Hembree 1979). Reciprocal cross-exposures indicated that the form from mosquitoes in Thailand was probably a single species. It was frequently found in both host species collected in the same locality. Its relationship to other helicosporidia is unknown. Because it occurs naturally in and can be transmitted to *Cx. quinquefasciatus*, the form from Thailand is likely to be different from the form in *Cx. nigripalpus*. However, there are few characters for morphological differentiation. Resolution of the relationships among the Helicosporida may require the application of techniques of protein chemistry or immunology.

METHODS AND MATERIALS

A colony of *Ae. aegypti* was maintained to provide hosts for the routine production of spores of the Thai helicosporidian and to provide larvae as experimental hosts. These larvae were used in dose-response type experiments to elucidate several biological characteristics of the helicosporidian and to assess the effects of various treatments on the infectivity of its spores.

MAINTENANCE OF HOST COLONY. The *Ae. aegypti* used were a US strain that has been maintained for several years at the US Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, MD. Larvae were reared at a water temperature of 26–27° C in plastic trays 56 × 43 × 13 cm, containing 5–6 liters of dechlorinated tap water. They were fed a 1/1/1 mixture of yeast, liver powder and ground high protein content hog chow. Under these conditions, first pupae appeared during the fifth day post-hatch, and pupation was mostly completed during the succeeding 2–3 days. Mortality during rearing was negligible, and aeration of the water was unnecessary. Adults were maintained in 60 × 60 × 60 cm screened wire cages at 25°C, RH 80% with a 12 hr photophase/12 hr scotophase. Guinea pigs were provided 3 times weekly for blood meals. Eggs were collected on paper toweling in beakers containing sufficient water not to evaporate for 1 wk. Egg papers were removed weekly, air dried and stored at room temperature. An excess was maintained so that all experiments could be done with larvae from eggs less than 1 mo old. Hatching was accomplished by placing sections of egg-paper in deoxygenated water containing a small amount of food for 30 min and subsequently placing the hatch container under a vacuum of about 17 psi for 30 min. The hatch period was kept to 1 hr to assure larvae of homogeneous age.

MAINTENANCE OF PATHOGEN CULTURE. Spores of *Helicosporidium* sp. from *Ae. aegypti* in Bangkok, Thailand, were shipped on dry ice from the Department of Medical Entomology, US Army Medical Component, Armed Forces Research Institute of Medical Science, Bangkok, Thailand. The pathogen had been maintained in that laboratory for about 2 yr in a Thai strain of *Ae. aegypti*. It has subsequently been maintained in this laboratory for almost 4 yr in a US strain of *Ae. aegypti*. There was no difference in susceptibility of these 2 strains of the host. Transmission was routinely achieved in 50 ml glass beakers by exposing groups of 100 24 hr old larvae in 20 ml total volume of a suspension of 10^3 spores/ml for 24 hr. Inoculum was prepared by triturating infected material in a Ten Broeck type tissue grinder, quantifying the spores with a hemocytometer and making the appropriate dilutions. A small amount of food was provided during exposure. Prior and subsequent to exposure, larvae were kept under uncrowded rearing conditions to avoid retarding their development. Ecdysis to the second larval stage commonly occurred during the 24 hr exposure period. Exposures were terminated by pouring the contents of each exposure container onto an organdy cloth screen

and gently rinsing the larvae with tap water to separate spores from larvae. The exposed larvae were then transferred to enameled pans containing 1 liter of dechlorinated tap water and reared as the stock colony but in a separate insectary. Infection rates near 100% usually resulted from these exposures. Infections retarded development. First pupation in infected larvae was delayed by a day or two, and pupation was protracted. Many individuals did not pupate. Mortality was insignificant during the first 5 days after initiation of exposure at this concentration, but it increased rapidly after that. The fat body was the primary tissue affected. Live pupae were discarded. Dead larvae and pupae were used as a source of inoculum for routine pathogen production. Spores used in experiments were harvested from 10–14 day old live infected larvae and pupae. Infected mosquitoes were not kept beyond 2 wk, by which time most individuals had either pupated and been removed or had died.

METHODS OF EXPERIMENTAL EXPOSURE AND DETERMINATION OF PERCENT TRANSMISSION. Experimental exposure of larvae was achieved by the same basic methods as used for routine pathogen production, except that in some experiments either the spores used or the hosts used or the method of exposure was manipulated as the experimental variable. Concentrations were quantified immediately before larval exposure and after whatever treatment the spores received. Exposure termination and post-exposure rearing were as for routine pathogen production. Percentages of transmission were determined by examining smears of larvae and/or pupae from control and experimental groups. Smears were made by placing specimens on paper towels to remove excess moisture, transferring them with fine tipped forceps to microscope slides, and using the tips of wooden applicator sticks to crush the specimens and smear them into an area of about 1 cm diam for fourth stage larvae or pupae. Proportionally smaller smears were made for younger material. Up to 5 smears were placed on each slide. Smears were air dried, fixed with methanol and stained for 1 hr with 2% Giemsa stain in 0.01 M phosphate buffer, pH 7.2. Coverslips were affixed with a synthetic mounting medium. Smears were examined at 650 X magnification with a Zeiss Photomicroscope. It was empirically determined that virtually all infections could be detected 4 days after the initiation of exposure by 2 passes across the smears reading adjacent fields of view. Older infections often could be detected in a single field because of the density of immature forms and spores. Infections as young as a few hours could be detected by examining whole smears, but this was exceedingly laborious. Examining 50 smears of larvae and/or pupae randomly selected from each group provided highly precise results from any given group. Routinely, unexposed larvae from the hatch used in an experiment were reared in parallel with larvae used in dose-response studies. These were examined along with the experimental material to confirm the absence of prior infections. When pathogen spores were receiving some treatment as the experimental variable, untreated spores were used as controls to confirm the normal infectivity of the treated spores prior to treatment.

EFFECT OF DURATION OF EXPOSURE ON PERCENT TRANSMISSION. Groups of 100 48 hr old *Ae. aegypti* larvae were exposed to 5 concentrations of helicosporidian spores varying from 500 to 5×10^4 spores/ml for each of 8 periods of exposure varying from 1 to 48 hr. Percent transmission was determined for each of the 40 experimental groups.

EFFECT OF HOST AGE AT EXPOSURE ON PERCENT TRANSMISSION. Five replicates, each of 100 larvae, were made for the 24 hr exposure of 2, 24, 48 and 72 hr old *Ae. aegypti* larvae to each of 5 concentrations. Two hr old larvae were exposed to concentrations from 25 to 500 spores/ml, 24 hr old larvae to concentrations from 50 to 10^3 spores/ml, 48 hr old larvae to concentrations from 10^3 to 5×10^4 spores/ml and 72 hr old larvae to concentrations

from 5×10^3 to 2.5×10^5 spores/ml. These age groups corresponded closely to first, second, third and fourth larval stages, respectively. Percent transmission was determined for each of the 5 replicates of each age/dose group. The 5 transmission rates determined for each age/dose group were averaged. Using these averages, regression lines of concentration on transmission were eye-fitted on semi-log graph paper, and IC_{50}s for this helicosporidian for each larval stage of *Ae. aegypti* were estimated.

OPTIMIZATION OF SPORE PRODUCTION. A reliable and plentiful supply of helicosporidian spores was essential to culture maintenance and to experimentation. Spore production in terms of total number of spores produced per exposed group was optimized relative to the spore concentration used to establish infections and to the time of harvest of the spores. Fifteen groups of 100 48 hr old larvae were exposed for 24 hr to each of 5 concentrations of spores. Concentrations varied from 500 to 5×10^4 spores/ml. For rearing subsequent to exposure, the 15 rearing pans in each dosage group were divided into 3 subgroups of 5 rearing pans (500 larvae) each. Spore production in these 3 subgroups was determined for each dosage group after 1, 2 and 3 wk, respectively. After 4 days, 20 larvae from each dosage group were collected, smeared and stained to estimate the percent of infection resulting from each dose. All live pupae and all dead larvae and pupae were collected daily and accumulated specifically for each subgroup of each dosage group. These were held at 4° C. At the end of 1, 2 or 3 wk all live larvae remaining in the appropriate subgroup of each of the dosage groups were collected, counted and combined with the previously collected material from that subgroup. The total number of specimens was determined and the probable number of infected specimens was calculated using the determined percents of infection. All were triturated, and the number of spores produced in each dosage group after 1, 2 and 3 wk was determined and expressed as total number of spores produced per time/dose group and average number of spores produced per infected individual.

EFFICIENCY EVALUATION. The purpose of these experiments was to answer the question: What concentration of spores, exposed to larvae of a given age, will result in what level of mortality in the exposed population by what time. A large number of *Ae. aegypti* larvae were hatched and transferred to rearing pans as if for normal rearing under uncrowded conditions with adequate food. At hatching and at the beginning of each of the 3 succeeding 24 hr periods of larval life, segments of the population were withdrawn for dose-mortality testing. Effectively, the dose-mortality response was tested for each successive larval stage. The exposure duration for each test was 24 hr. The first stage was tested against 7 concentrations from 50 to 5×10^4 spores/ml, the second stage against 7 concentrations from 100 to 10^5 spores/ml, the third stage against 6 concentrations from 10^3 to 5×10^5 spores/ml and the fourth stage was only tested against concentrations of 10^5 and 5×10^5 spores/ml. Five groups of 100 larvae composed each dosage group for each instar. These were exposed and reared as described above. Five groups of 100 larvae from the same hatch, unexposed but otherwise treated exactly the same as the exposed larvae, were maintained for each instar to provide a control mortality curve. Daily, beginning at the termination of exposure, all dead specimens were counted and removed from the experiment. Live pupae were held in separate cages for each age/dose group, and dead pupae and adults were counted and removed daily. Cumulative percent mortality was determined. The experiment was continued for 28 da after the initiation of exposure. It was repeated 3 times. The data were accumulated and used to plot curves of cumulative mortality against age of mosquito for spore concentrations used against each instar.

STORAGE PROPERTIES OF *Helicosporidium* SP. Large quantities of helicospo-

ridian spores were washed 3 times for 10 min at 1000 × g in demineralized water. (All washes were at 1000 × g for 10 min.) The washed spores were used in experiments to determine the effects of various methods of storage on the infectivity of the spores. Some spores were held in 0.05 M phosphate buffer, pH 7.0, at room temperature, in the dark. Their infectivity to 48 hr old larvae was determined at 10 and 17 days using concentrations varying from 5×10^3 to 3×10^5 spores/ml. Some spores were held in demineralized water at 4° C. Their infectivity to 48 hr old larvae was determined periodically between 1 and 16 wk using concentrations between 5×10^3 and 7×10^4 spores/ml. Some spores were quick frozen in dry ice-acetone in either demineralized water or in 10% glycerin in water or 10% dimethyl sulfoxide (DMSO) in water as cryoprotectants and held at −70° C for 6 months. Their infectivity to 24 hr old larvae was determined using concentrations varying from 10^3 spores/ml to 10^4 spores/ml. Some spores were resuspended in a minimum volume of demineralized water and transferred to lyophilization ampoules. They were either quick frozen in dry ice-acetone and lyophilized or simply vacuum dried. These were held at room temperature in the dark for 4 wk, after which their infectivity to 48 hr old larvae was determined using concentrations varying from 500 to 5×10^5 spores/ml.

EFFECTS OF ENVIRONMENTAL FACTORS ON SPORE INFECTIVITY. Numerous factors in the aquatic environment might be expected to influence pathogen spore survival and infectivity. Among these are temperature, pH, salinity, pollutants and ultraviolet (UV) light. Tests were conducted to assess the effects of water temperature, pH as provided by several different buffer systems, sodium chloride, detergent and UV light on spore infectivity. Determination of percent transmission resulting from exposure of 24 hr old larvae for 24 hr to spores subjected to various treatments constituted the test system. Prior to specific treatments, spores were washed 3 times in distilled water. Following treatment with chemicals, spores were washed twice to terminate exposure. A single group of 100 larvae was exposed to each of 5 concentrations varying from 50 to 5,000 spores/ml, of spores receiving each separate treatment. Percent transmission was determined by examining 50 of these larvae collected from each group 4 da after initiation of larval exposure.

To determine the effects of temperature, larvae were exposed to spores that had been held at 25, 32, 37, 42 and 50° C for 1, 2, 4, 6 and 24 hr. Additional groups of spores were subsequently treated at 50° C for 15, 30, 45, 60 and 75 min. Spores were treated while in 2 ml of distilled water held in water baths at the indicated temperatures for the times shown. Control spores were held at 4° C for 24 hr.

To assess the effects of several buffer/pH combinations on spore infectivity, larvae were similarly exposed to spores that had been held for 24 hr at 4° C in distilled water (control) or for 24 hr at 4° C in: 0.05 M carbonate-bicarbonate buffer, pH 10.5; 0.05 M boric acid-borax buffer, pH 8.4; 0.05 M phosphate buffer, pH 7.1; 0.05 M cacodylate buffer, pH 5.0; and 0.05 M citrate-phosphate buffer, pH 3.0.

To assess the effects of saline and a common liquid household dish washing detergent on spore infectivity, spores were exposed to 5 and 10% saline (w/v) and to 5 and 10% detergent (v/v) for 24 hr at 4° C.

For exposure to UV light, spores were pipetted onto a millipore filter that was kept moist by placing it on a moistened paper pad in an open 15 cm petri dish. The light source was a G15T8 15 watt General Electric germicidal tube placed 20 cm above the spores for a period of 1 hr. Following exposure to the UV light, spores were washed from the filter, quantified, and diluted to the concentrations used.

PRODUCTION OF MOSQUITO HELICOSPORIDIAN SPORES IN FALL ARMYWORM. Current technology does not permit the

economical mass production of parasitic protozoans in artificial media. The most prospective approach to producing large quantities of spores of protozoan insect pathogens is to produce them in mass producible alternative hosts such as lepidopteran larvae. Larvae of the fall armyworm, *Spodoptera frugiperda* (Smith), were used as test insects in which to demonstrate the infectivity of the mosquito helicosporidian to Lepidoptera larvae. The susceptibility of *Ae. aegypti* to spores produced in these larvae was then compared with that of spores produced by *Ae. aegypti*. Helicosporidian spores from *Ae. aegypti* were washed 3 times in saline and then placed on discontinuous gradients of 20, 40, and 60% Ludox HS40, a colloidal silicate material with pH of about 9.4. Gradient centrifugation was at 15,000 rpm for 15 min in a Beckman 21 rotor at 4° C. Spores appearing to be free of debris by microscopic examination were recovered from the top of the 60% Ludox. Preliminary observations indicated that some bacteria would remain with the spores. So, the spores were washed 3 times in saline containing 500 μg/ml Neomycin, which had been shown to be nontoxic to spores at this concentration, to dilute out the Ludox and suppress bacterial growth. The final pellet was resuspended in the same solution. A microapplicator was used to inject 10^4 spores in 5 microliters volume into each of 100 2.5 cm long armyworms in the mid-dorsal region in an area dabbed with 70% ethanol. After 14 days, the surviving armyworms were triturated one at a time in Ten Broeck type tissue grinders. Presence or absence of spores was determined. The triturants of infected specimens were accumulated, spore production was quantified, and the spores were washed 3 times in distilled water to reduce the amount of debris present. The infectivity of these spores was then tested at concentrations varying from 100 to 10^4 spores/ml in comparison with helicosporidian spores freshly recovered from infected mosquito larvae.

INFECTIVITY TO OTHER MOSQUITO SPECIES. Using the same methods used for experiments in *Ae. aegypti*, the infectivity of this *Helicosporidium* sp. was determined for 48 hr old laboratory reared *Anopheles dirus* Peyton and Harrison larvae (second stage) using concentrations from 10^3 to 10^4 spores/ml and for 48 hr old laboratory reared *An. maculatus* Theobald larvae (second stage) using concentrations from 7.5×10^3 to 15×10^3 spores/ml. Infectivity of the helicosporidian for 24 hr old laboratory reared *Ae. taeniorhynchus* (Wied.) larvae (first stage) was determined using concentrations from 100 to 10^4 spores/ml. The infectivity of the helicosporidian for laboratory reared *Toxorhynchites splendens* (Wied.) was tested by 2 methods. First and third stage larvae were exposed to 10^6 spores/ml for 24 hr. Third stage larvae were also exposed by allowing them to feed at will for 5 successive days on known helicosporidian infected *Ae. aegypti* larvae.

RESULTS AND DISCUSSION

Repeated examinations of control groups confirmed the absence of helicosporidian infection in the stock colony and the reliable infectibility of that colony with spores of the helicosporidian. Dose-infectivity results, acquired under the same experimental conditions but at different times with different batches of larvae and spores, naturally varied, since 2 variable, living systems were brought together in these experiments under conditions that were imperfectly constant.

EFFECTS OF DURATION OF EXPOSURE ON PERCENT TRANSMISSION. In Table 1 it is shown that percent transmission was directly proportional to both duration of exposure and to concentration of spores in the exposure medium. The lowest concentration that resulted in infection of all exposed specimens was 5×10^3 spores/ml, regardless of duration of exposure. Spore concentrations of 5×10^4 spores/ml produced 100% transmission in only 1 hr, while a 4 hr exposure was required to produce infection in all specimens at 10^4 spores/ml.

Table 1. Percent transmission of *Helicosporidium* sp. to 48 hr old *Aedes aegypti* larvae at different spore concentrations and durations of exposure.

Concentrations (spores/ml)	Durations of exposure (hours)							
	1	4	8	16	24	32	40	48
500	4	4	6	6	8	10	10	12
1,000	6	10	14	20	24	38	32	36
5,000	46	72	80	88	88	95	100	100
10,000	94	100	100	100	100	100	100	100
50,000	100	100	100	100	100	100	100	100

EFFECTS OF HOST AGE AT EXPOSURE ON PERCENT TRANSMISSION. As indicated in Table 2, the age of larvae at exposure has a dramatic effect on susceptibility. Mean infection rates within each age group increased in direct proportion to dose. The estimated IC_{50}s were 500, 1500, 100,000 and 500,000 spores/ml for first, second, third and fourth stage larvae, respectively. Although experiments were not specifically conducted to demonstrate it, it is felt that the important variable is biological age, not chronological age. Larvae that were 72 hr old, but which were only in the second stage as a result of being maintained at cool temperatures, would be just as susceptible as 24 hr old second stage larvae, if both were exposed under the same conditions.

OPTIMIZATION OF SPORE PRODUCTION. To produce spores, a larva must be infected and it must remain alive long enough to permit pathogen multiplication and maturation. It is preferable that they remain in an immature stage to minimize handling and avoid the scales of adult mosquitoes which complicate spore recovery and quantification. Younger larvae are easier to infect, as shown above, but as expected they succumb more easily to infections. Conversely, if they live, they might be expected to produce more spores by a certain age, having additional time for spores to develop.

Larvae 48 hr old were selected for this experiment as a compromise between the fragility of younger larvae and the difficulty of infecting older ones. As can be seen in Table 3, many fewer spores were produced in only 1 wk than in 2 or 3 weeks. There was little difference between groups held 2 wk and those held for 3 wk. It appeared that the largest total number of spores and the largest number of spores per infected larva were produced in larvae held for 2 wk, only because nearly all larvae were dead by that time, or had pupated, if uninfected. The largest total number of spores produced in the 2 wk group was more than twice the number produced by any group held only 1 wk, justifying the additional cost and use of lab space. There seems to be no justification in holding cultures for the third week. The largest total number of spores produced in any group was at a spore concentration resulting in slightly less than 100% infection. This suggests that the greatest number of spores might be produced at the minimum spore concentration that would produce 100% infection. This concentration should minimize the virulence of infections which tends to reduce the total number of spores produced by causing premature mortality. If this concentration could be defined for still younger larvae, and if these survived well till harvest time, spore production might be increased. With the intent of increasing spore production in our routine pathogen production system, we reduced the age of larvae at exposure to 24 hr and the concentration to 10^3 spores per ml, the minimum concentration that caused acceptable levels of infection in larvae of that age. Our production is now 3.1×10^5 spores per infected larva.

Spore production in *Ae. aegypti* in the laboratory has never approached the

Table 2. Percent transmission of *Helicosporidium* sp. to *Aedes aegypti* larvae exposed at different ages to different spore concentrations. Duration of exposure was 24 hr.

2 hr old larvae								24 hr old larvae							
Concentration (spores/ml)	Replicate 1	2	3	4	5	$\bar{x}$	s.d.	Concentration (spores/ml)	Replicate 1	2	3	4	5	$\bar{x}$	s.d.
25	4	8	0	0	0	2.4	3.6	50	12	4	16	4	8	8.8	5.2
50	12	4	12	4	8	8.0	4.0	100	8	12	16	12	25	14.6	6.5
100	12	10	28	24	16	20.0	6.3	250	20	28	16	20	16	20.0	4.9
250	36	32	28	28	32	31.2	3.3	500	28	24	32	28	20	26.4	9.6
500	64	64	60	48	40	55.2	10.7	1,000	60	68	60	76	52	63.2	9.1
48 hr old larvae								72 hr old larvae							
Concentration (spores/ml)	Replicate 1	2	3	4	5	$\bar{x}$	s.d.	Concentration (spores/ml)	Replicate 1	2	3	4	5	$\bar{x}$	s.d.
1,000	0	0	0	0	0	0	0	5,000	0	0	0	0	0	0	0
5,000	0	0	0	0	0	0	0	10,000	0	0	0	0	0	0	0
10,000	24	16	10	4	16	16	8.6	50,000	0	0	0	4	0	0.8	1.8
25,000	36	28	28	36	32	32	4.0	100,000	20	8	12	16	12	13.6	4.6
50,000	40	36	40	36	48	40	4.9	250,000	28	52	28	40	40	37.5	10.0

Table 3. Number of *Helicosporidium* sp. spores produced in *Aedes aegypti* larvae exposed for 24 hr beginning at 48 hr of age to different concentrations of helicosporidian spores and subsequently held for 1, 2 or 3 weeks.

Concentrations (spores/ml)	Percent trans.	Infected larvae	After 1 wk		After 2 wk		After 3 wk	
			Spores produced	Spores/infected larvae	Spores produced	Spores/infected larva	Spores produced	Spores/infected larva
500	24	115	13.3×10^6	11.5×10^4	27.5×10^6	23.9×10^4	21.5×10^6	18.7×10^4
1,000	54	259	20.1×10^6	8×10^4	42.3×10^6	16.3×10^4	49.7×10^6	19.2×10^4
5,000	92	442	30.6×10^6	6.9×10^4	87.8×10^6	19.8×10^4	64×10^6	14.5×10^4
10,000	100	480	32.5×10^6	6.8×10^4	58.4×10^6	12.1×10^4	45.6×10^6	9.5×10^4
50,000	100	480	15.5×10^6	3.2×10^4	26.7×10^6	5.6×10^4	34.1×10^6	9.1×10^4

number of spores per larva found in field-collected *Cx. quinquefasciatus* in Thailand. Heavy infections with this pathogen in field-collected specimens of this host species produced identifiable gross signs of infection and permitted their selective collection and the determination that well over 10^6 spores per larva could be produced (Hembree 1979). Infections with this pathogen in *Ae. aegypti* produced no reliable signs. Therefore, this information was not acquired for field-collected *Ae. aegypti.* Quantitative spore production studies with *Cx. quinquefasciatus* in the laboratory have not been done. Thus, we do not know if the difference in spore production in these host species relates to host species differences or to habitat differences.

EFFICIENCY EVALUATION. The curves in Fig. 2 are plots of the cumulative mortality, combined for all stages, of *Ae. aegypti* exposed initially in each of the larval stages to various concentrations of helicosporidian spores. Data for alternate days were used to make the curves. Control mortality curves are also given for each experiment. The curves theoretically could be used to estimate spore concentrations that would be required to effect mortality of a desired level by the time a target vector population reached a particular age. Numerous other variables would influence results, if attempts were made to duplicate these observations in field populations. However, these kinds of data will be necessary in assessing the usefulness of pathogens that kill slowly by chronic, debilitating disease. Both time and level of mortality in these infections will be related to virulence, which will be related to the infecting dose, which will greatly influence the cost of production. As shown above, age of larvae at exposure is a primary determinant of the dose required to achieve infections.

STORAGE PROPERTIES OF *Helicosporidium* SP. Baseline dose-response studies with *Helicosporidium* sp. spores after washing but before storage indicated that washing had no noticeable effect on spore infectivity. Infectivity of both lyophilized and vacuum dried spores was effectively destroyed after 4 wk storage at room temperature, even when concentrations as high as 5×10^5 were used in exposures of 48 hr old larvae. A dramatic reduction in infectivity occurred in spores stored at room temperature in 0.05 M phosphate buffer, pH 7.0. After 10 days storage, only 28% infection was achieved in 48 hr old larvae at a spore concentration of 3×10^5 per ml. After 17 days storage this dropped to 6%. Spores held infectivity very well in demineralized water at 4° C, as indicated in Table 4. There was virtually no loss of infectivity to 24 hr old larvae resulting from quick-freezing followed by storage at −70° C for 6 mo for spores in the cryoprotectants, 10 percent glycerin and 10 percent DMSO. Spores quick-frozen in water and stored for 6 mo at −70° C retained no infectivity to 24 hr old larvae at concentrations up to 10^5 spores/ml.

EFFECTS OF ENVIRONMENTAL FACTORS

Table 4. Percent infection achieved in 48 hr old *Aedes aegypti* larvae exposed to spores of *Helicosporidium* sp. stored in demineralized water at 4° C for various times.

Concentrations (spores/ml)	Duration of storage (weeks)								
	0	1	2	4	6	8	10	12	16
5,000	86	90	90	90	88				
10,000	100	100	100	98	100	68	80	52	88
20,000	100	100	100	100	100				
30,000						100	100	90	100
50,000	100	100	100	100	100				
70,000						100	100	100	100

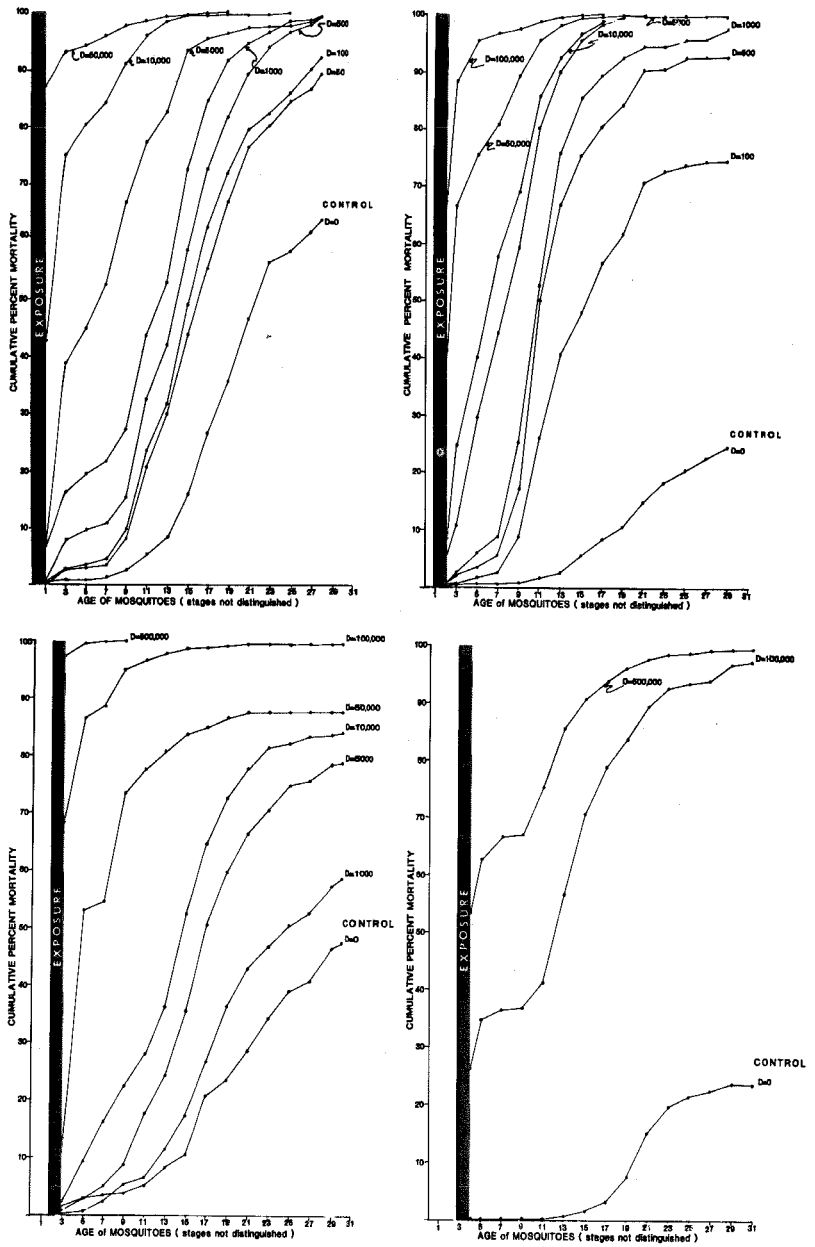

Fig. 2. Cumulative mortality occurring in laboratory exposed *Aedes aegypti* exposed to various concentrations of spores of *Helicosporidium* sp. as first, second, third and fourth stage larvae. D = Dose in spores/ml.

ON SPORE INFECTIVITY. As shown in Table 5, helicosporidian spores endured 25° and 32° C for up to 24 hr with virtually no loss of infectivity. Some loss of infectivity appeared to occur in spores exposed to 37° C for more than a few hours. Twenty-four hr exposure to 42° C caused significant decrease in infectivity. These figures indicate that spores should survive environmental temperatures long enough to effectively expose larvae present at application or hatching within at least the first 24 hr after application. Exposure to 50° C for even 15 min reduced transmission at the highest concentration (5×10^3 spores/ml) to only 4%. However, this offers the hope that spores might survive 50° C for the few seconds likely to be needed for their microencapsulation into special formulations.

Spores demonstrated no noticeable loss of infectivity in buffer solutions with pH ranging from 10.5 to 3.0 at 4° C for 24 hr. These are conservative data, since treatment of spores at 4° C could be expected to be less detrimental than exposure under field conditions. However, this offers the hope that spores will be relatively unaffected by the pH encountered in most mosquito producing aquatic habitats.

As indicated in Table 6, 5 and 10% household detergent had only minor effects on infectivity of helicosporidian spores. Five percent saline reduced transmission. Ten percent saline and intense exposure to UV light destroyed or dramatically reduced infectivity of spores. These data also are conservative, since spores were treated at 4° C.

PRODUCTION OF MOSQUITO HELICOSPORIDIAN SPORES IN FALL ARMYWORMS. Only 12 of 100 injected fall armyworms survived for 14 days. Whether this high mortality resulted from bacterial contamination during injection or from helicosporidian infections could not be determined. Most died in the process of pupation. The specimens remaining alive had pupated. All were infected and produced an average of 2×10^7 helicosporidian spores. This infection rate and high

Table 5. Percent infection resulting from 24 hr exposure of 24 hr old *Aedes aegypti* larvae to *Helicosporidium* sp. spores that had been exposed to selected temperatures for various periods of time.

Concentration (spores/ml)	Temperatures and durations of exposure (hr)																					
	25° C					32° C					37° C					42° C					
	C	1	2	4	6	24	C	1	2	4	6	24	C	1	2	4	6	24	C	1	2	4	6	24
50	32	20	20	24	28	32	40	46	76	52	72	56	40	24	28	32	32	32	36	40	36	28	12	0
100	44	28	40	32	36	48	60	60	64	84	80	80	48	36	40	36	48	48	50	56	60	50	28	10
500	84	68	48	64	76	68	84	84	100	100	100	96	80	68	80	64	56	56	78	74	70	68	60	8
1,000	88	80	64	76	84	72	100	100	100	100	100	100	85	80	80	76	72	60	92	90	86	84	86	18
5,000	100	100	92	100	100	100	100	100	100	100	100	100	100	92	88	80	76	80	100	100	100	100	98	32

Table 6. Percent transmission resulting from exposure of 24 hr old *Aedes aegypti* to *Helicosporidium* sp. spores exposed to saline, household detergent and UV light.

Concentration (spores/ml)	Control	Saline 24 hr @ 40° C		Detergent 24 hr @ 40° C		U/V light	
		5%	10%	5%	10%	1 hr	1 hr
50	32	20	0	40	18		0
100	50	24	0	48	40		0
500	80	48	0	84	64		0
1,000	100	88	0	86	80		8
5,000	100	92	0	100	96		12

spore count suggest the dead armyworms could have died as a result of helicosporidian infections.

The infectivity for 24 hr old *Ae. aegypti* larvae exposed for 24 hr to these spores was reduced from that of spores produced in the mosquito host. Concentrations of 100, 500, 1,000, 5,000 and 10,000 spores/ml from the armyworm resulted in transmission rates of 8, 12, 28, 40 and 60% respectively, whereas the same concentrations of spores from mosquitoes produced transmission rates of 24, 92, 100, 100 and 100% respectively. Despite the apparent reduction in infectivity, there appears to be a possibility that this pathogen could be produced in a mass-producible lepidopteran larva with resulting high spore yields.

INFECTIVITY TO OTHER MOSQUITO SPECIES. The *Helicosporidium* sp. was transmitted easily to several other mosquito species (Table 7). Transmission to *An. stephensi* Liston and to *An. minimus* Theobald has been confirmed but not quantified. Third stage *Tx. splendens* were refractory to *per os* exposures at 10^6 spores/ml, but 25% of first stage larvae could be infected at that dose. Four percent of third stage larvae were infected when allowed to feed to repletion on infected *Ae. aegypti* larvae for 5 days.

This helicosporidian was infectious to several species of medically important mosquitoes. It appeared to be sufficiently resistant to some common environmental stressors to survive long enough in nature to effect transmission. Adequate spore concentrations resulted in high levels of control in the laboratory, though possibly not quickly enough to interfere with transmission of some diseases in the field, if application was delayed until larvae were in their fourth stage. The doses required to effect mortality quickly in older larvae may be prohibitively high. It appears that it could be produced in large quantities in an alternate host, but I have serious reservations that production by such methods would be cost-competitive or practical, considering the poor survival of the pathogen spore for long periods of time without special care in storage, such as refrigeration or freezing. Methods of production in artificial media are needed, because they would be easier to automate and hopefully less expensive. Failure to survive long at ambient temperatures in water or to survive drying make it diffi-

Table 7. Percent transmission occurring in *Anopheles dirus, An. maculatus* and *Aedes taeniorhynchus* after 24 hr exposures to spores of *Helicosporidium* sp.

Concentration (spores/ml)	*An. dirus* (48 hr old)	*An. maculatus* (48 hr old)	*An. taeniorhynchus* (24 old)
500	—	—	38
1,000	60	—	56
5,000	96	—	92
7,500	—	10	—
10,000	100	—	100
11,250	—	22	—
15,000	—	48	—

cult to consider this a serious candidate microbial control agent. Microencapsulation might contribute to survivability, but it also would increase cost. The finding of a helicosporidian in *Daphnia* will cause apprehensions about its danger to other aquatic invertebrates until the helicosporidia and their host ranges are better known (Sayre and Clark 1978). However, the organism is easily maintained in the laboratory and should make a good model for a variety of basic studies.

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