THE ISOLATION OF MICROSPORIDIA AND OTHER PATHOGENS FROM CONCENTRATED DITCH WATER

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ABSTRACT. Water from a mosquito larval habitat in Florida was collected periodically for one year. After removing debris and macroscopic organisms, the small particles were concentrated by continuous flow centrifugation and examined microscopically. *Anopheles quadrimaculatus, Culex salinarius* and *Heliothis zea* larvae were exposed to the concentrates. The microsporidia isolated were *Nosema, Pleistophora, Telomyxa, Vavraia* and *Vairimorpha.* In addition to these microsporidia, a *Helicosporidium* (Protozoa), a *Metarrhyzium* (fungi) and two cytoplasmic polyhedrosis viruses were also isolated.

INTRODUCTION

The search for pathogens of potential utility for biological control is generally conducted by examining large numbers of insects for signs of disease. A wide variety of microsporidia are readily found in insects. Aquatic insect larvae such as mosquitoes, however, are infected by many microsporidia which have an intermediate host (Andreadis and Hall 1979, Andreadis 1983, Sweeney et al. 1985) or transovarial transmission (Hazard and Weiser 1968, Sprague 1977) and the spores found in the insects are not infectious to the host from which they come. There is also no guarantee, because of transovarial transmission, that the stages infectious to the larvae are present when infected larvae are found. There could therefore be spores in the larval habitat infectious to mosquito larvae, even in the absence of mosquito larvae, or at such low concentrations that larvae present failed to become infected.

This study examines the possibility of isolating microsporidia and other pathogens with resistant infectious stages in an aquatic habitat by screening water samples. To enhance the probability of infecting the target mosquito and lepidopterous hosts, the water is processed through a continuous flow centrifuge to concentrate the spores which might be present.

MATERIALS AND METHODS

Water Collection: The water samples were obtained from a ditch in a small woodland area in Gainesville, FL, on the USDA-ARS Laboratory compound (1700 SW 23rd Drive). The ditch was under a canopy of longleaf pine (*Pinus palutris* Mill.) and sweetgum (*Liquidambar styraciflua* L.) and is approximately 2 m wide with a dense layer of leaf litter on the bottom. The ditch is confluent with a shallow runoff pond, and its depth, which varies with the amount of rainfall, is between 0 and 30 cm. Mosquito larvae and their microsporidia at this site have been monitored since 1969 by several researchers at the Insects Affecting Man and Animals Research Laboratory (E. I. Hazard, S. W. Avery, D. W. Anthony, A. H. Undeen).

Samples of between 11 and 16 liters of water were collected during 1983 and 1984 from a 10-m portion of the ditch and strained through a series of sieves. Invertebrates from the samples were set aside for further examination. The water was further filtered through non-woven interfacing and the microscopic contents concentrated by continuous flow centrifugation at 10,000 rpm into 50-ml tubes in a refrigerated centrifuge (5 ± 1°C). The sediment from the tubes was combined and resuspended in 15 ml of deionized water. Total concentration of all microsporidian spores was determined by hemacytometer counts with phase contrast microscopy. A random sample of spores in the final suspension was measured with an image-splitting eyepiece.

The invertebrates from the water were crushed and examined for microsporidia spores, and when found these spores were fed to *Heliothis zea* Bodie, *Anopheles quadrimaculatus* Say and *Culex salinarius* Coquillett as described for the spores concentrated from the water samples.

To prevent contamination from laboratory cultured spores, the continuous flow apparatus and all other equipment, which contained the water samples or concentrates, was sterilized prior to use. As a control against contamination, 5 liters of deionized water was passed through the continuous flow system. The bottom 2 ml of the water in the collecting tubes was examined for spores. First instar *An. quadrimaculatus* larvae were exposed to this sample of water in a total volume of 10 ml for 24 h, then transferred to 500 ml of well water and reared to adults and examined for infection.

Experimental Host Exposures: First instar larvae of *An. quadrimaculatus* and *Cx. salinarius* were exposed to 10, 20 or 40% suspensions of the ditch water concentrated sediments in 10-

ml volumes for 24 h, and then transferred to 500 ml of water and reared at 27°C. When these larvae reached the third or fourth instar, they were examined against a black background for visible signs of infection. After the adults emerged and mated, males were crushed under coverslips to look for spores and also examined as Giemsa-stained smears. Females were offered a blood meal and isolated for oviposition, after which they were examined for infections as described for the males. The larvae of the F_1 generation were reared to adults and examined as described above.

Heliothis zea larvae (5 days old) were starved in individual containers for 24 h and then provided with 0.05 ml of field water concentrate. Twenty-four hours later they were transferred to individual rearing containers. Pieces of tissue from dead larvae were crushed under coverslips and examined for spores. Adults were examined this way for spores, and smears were prepared, stained with Giemsa's stain, and examined for developmental stages of microsporidia. Spores from infected individuals were washed and saved in deionized water at 8°C.

RESULTS

Microsporidia Spores in the Samples: Microsporidia spores in the concentrate were recognized by their shape and refractile appearance in a phase contrast microscope. Only *Telomyxa* sp. was readily identified in the water samples. The volume of water per sample and the estimated spore concentration in the ditch water are presented in Table 1. Fewest spores were found during August and September when rainfall was greatest and the water levels were highest (13.7 cm just before the September collection) and the invertebrate fauna was the most impoverished. The highest spore concentrations were found from October–January. Most of these spores were small, less than 2.5 μm long. From March through May, the spore concentrations were less than 1,000/ml, but the spores were more variable in size and morphology. The mean (± SE) pH of the water was 6.5 ± 0.1.

Many invertebrates, including four genera of mosquitoes (*Aedes* spp., *Anopheles* spp., *Culex* spp. and *Uranotaenia* spp.) were collected from the site. Their numbers were generally low, and no symptoms of disease were observed in any of the mosquito larvae. Mosquito larvae are, therefore, an unlikely source for the spores infective for the *H. zea* and mosquitoes in the lab. In other organisms collected, several microsporidioses were observed (Table 2); however, only the *Telomyxa* sp. from helodid larvae (*Ora texana* Champ.) (Hazard and Federici 1985), which has a distinctive spore morphology, was readily identified in the water samples. These spores occur in pairs and measured 6.9 ± 0.1 × 3.0 ± 0.1 μm. Infected helodid larvae (Coleoptera) were collected during September and November; however, *Telomyxa* spores first appeared in the water in August and were present in the samples until December. In October some of the paired *Telomyxa* spores had one germinated spore, whereas in November most of the paired spores in the samples were germinated. Mature *Telomyxa* spores were observed again in April and May.

Table 1. Volume of water sampled and estimated spore concentrations in the ditch water samples.

Collection date	Sample volume (liters)	No. of spores/ml of field water
May 11, 1983	11.0	—[a]
June 2, 1983	12.0	900
Aug. 8, 1983	13.5	269
Sep. 23, 1983	15.0	70
Oct. 5, 1983	13.8	1500
Oct. 18, 1983	13.2	1800
Nov. 1, 1983	14.6	430
Dec. 6, 1983	14.5	3000
Jan. 4, 1984	14.2	2700
Jan. 26, 1984	14.0	2100
Mar. 5, 1984	13.6	719
Mar. 26, 1984	16.0	332
Apr. 16, 1984	13.0	311
May 1, 1984	13.9	200

[a] Not determined.

Table 2. Collection of aquatic organisms infected with microsporidia.

Host	Pathogen	Collection dates
Helodidae	*Telomyxa*	Sep. 23–Nov. 1, 1983; July 12, 1984
Dytiscidae	*Pleistophora* sp.	Mar. 26, 1984
Odonata	*Pleistophora* sp.	Oct. 5, 1984
Amphipod	*Octosporea* sp.	Aug. 8–Oct. 18, 1983; Mar. 16, 1984; Jul. 12, 1984
Daphnia sp.	Thelohaniidae	Sep. 22, 1983
Daphnia sp.	*Nosema* sp.	Jan. 28, 1984; Mar. 26–Apr. 16, 1984; June 4, 1984
Collembola	*Nosema* sp.	Jan. 28–May 1, 1984
	Thelohaniidae *Helicosporidia* sp.	

Infections in Experimental Hosts: Examination by phase microscopy of the distilled water which had been passed through the continuous flow system revealed no microsporidia spores. Additionally, no infection occurred in the *An. quadrimaculatus* exposed to the bottom 2 ml of

the water, indicating that infections were not caused by spore-contaminated equipment.

Nosema: Of the 14 water samples, 5 of them produced a *Nosema* infection in *An. quadrimaculatus*, 3 in *Cx. salinarius* and 6 in *H. zea* (Tables 3, 4 and 5). In November, 5% of the *H. zea* larvae were infected, whereas no infection was observed in the mosquitoes exposed to the same water sample. None of the aquatic organisms collected with the water samples which induced the *Nosema* infections was found infected with *Nosema*. The spores from laboratory infected hosts were 3.56 ± 0.05 × 1.99 ± 0.19 μm. Spores of the same size and shape were present in most of the collections and accounted for 40 to 50% of the spores in the samples collected in June and December 1983. These were the same collections which induced the highest *Nosema* infection rates in the target hosts.

Vavraia: *Vavraia* sp. also infected each of the experimental hosts. In May 1983 all three hosts were infected. However, *Vavraia* only infected *H. zea* in June and November 1983, when the percentage of spores within the *Vavraia* size range in the water sample was 38 and 41%, respectively. These spores measured 4.37 ± 0.08 × 2.51 ± 0.03 μm.

Vairimorpha: *Vairimorpha* spp. was the most common microsporidian isolated in *H. zea*. Spores in its size range were present in 67% of the water samples. The average infection rate was 20%, with the highest rates occurring in November and May (70 and 67%, respectively). The lowest infection rate occurred in December (6%). *Vairimorpha* spp. was not isolated in the samples collected in late summer (August and September) or in January and March. The *Vairimorpha* isolated in November were easily identified because both free spores and octospores were found in the *H. zea* exposed to the ditch water. In others the identification was confirmed only after a second *per os* transmission in *H. zea* when octospores were observed in larvae reared at 20°C. None of the *Vairimorpha* isolated were infectious to mosquitoes.

Pleistophora: *Pleistophora* sp. was isolated in *H. zea* from 36% of the water samples, with an average infection rate of 4%. The spores measured 2.6 ± 0.06 × 1.21 ± 0.02 μm and were recovered from adults in three of the tests and once from a moribund larva. Spores with this size range were found in all of the water samples and accounted for 25–31% of the spores in those samples which induced infections in *H. zea*. The infections appeared to be in the fat body of the *H. zea*.

Table 3. Infections in *Anopheles quadrimaculatus* exposed to concentrated ditch water samples.

Collection date	Pathogen	Percent infection
May 11, 1983	*Nosema* sp./ *Vavraia* sp.	28.6
June 2, 1983	*Nosema* sp.	46.73
Sep. 23, 1983	*Nosema* sp.	<5.03
Oct. 18, 1983	*Telomyxa*	4.5
Dec. 6, 1983	*Nosema* sp.	23.13
Mar. 5, 1984	*Nosema* sp.	13.50
Apr. 16, 1984	CPV	13.6
May 1, 1984	CPV	8.3

Table 4. Pathogens in *Culex salinarius* exposed to concentrated ditch water samples.

Collection date	Pathogen	Percent infection
May 11, 1983	*Nosema* sp./ *Vavraia* sp.	44.4 —[a]
June 2, 1983	*Nosema* sp.	14.3
Mar. 6, 1984	*Nosema* sp.	3.1
	CPV	31.3
Mar. 26, 1984	CPV	25.0
Apr. 16, 1984	CPV	4.5
May 1, 1984	CPV	8.3

[a] Mixed infection of the two pathogens.

Table 5. Pathogens in *Heliothis zea* exposed to concentrated ditch water samples.

Collection	*Nosema*	*Vavraia*	*Vairimorpha*	*Pleistophora*	*Metarrhyzium*	*CPV*
May 11, 1983	50.0	—[a]	6.3	3.1	0	0
June 2, 1983	60.0	30.0	0	0	0	0
Sep. 23, 1983	0	0	0	0	6.3	0
Oct. 5, 1983	0	0	15.8	0	5.3	47.0
Oct. 18, 1983	0	0	24.0	4.5	4.0	0
Nov. 1, 1983	5.0	10.0	70.0	0	6.0	0
Dec. 6, 1983	19.2	0	5.8	4.2	0	0
Jan. 4, 1984	0	0	6.3	3.1	0	18.8
Jan. 26, 1984	3.3	0	0	0	0	33.3
Mar. 5, 1984	16.7	0	0	0	4.2	12.5
Mar. 26, 1984	0	0	11.8	2.9	2.9	0
Apr. 16, 1984	0	0	3.3	0	0	0
May 1, 1984	0	0	68.8	0	6.3	0

[a] Mixed infection with *Nosema* sp.

Telomyxa: In October mature *Telomyxa* spores were at their highest concentration (150 spores/ml of original water). After exposure of *An. quadrimaculatus* to this sample, oblong free spores were found in 5% of the adults. These live spores measured 4.3 × 1.6 μm. *Anopheles quadrimaculatus* fed the disporous spores of *Telomyxa* sp. harvested from helodid larvae collected from the test site were also infected with a similar oblong free spore with an infection rate of 20%. These spores were morphologically dissimilar to the disporous spores found in the helodid larvae. The intensity of the infection was very low—estimated to be less than 200 spores/adult mosquito. Sporogony appeared to be complete so that the developmental stages of these spores were not observed in Giemsa-stained smears.

Helicosporidium: In May 1984, 19% of the *H. zea* exposed to the field water were infected with *Helicosporidium* sp. The spores were slightly flattened spheres 5.58 ± 0.03 μm in diameter as fresh spores and 4.44 ± 0.07 μm in Giemsa-stained smears. High anopheline mortality was observed 48–72 h postexposure, but *Helicosporidium* development was observed in less than 10% of the surviving larvae with low spore production in the infected individuals. No evidence of development was observed in *Cx. salinarius*. Collembola were the only invertebrates collected from this site with *Helicosporidium* infection. However, the spores from Collembola were larger, measuring 7.19 ± 0.15 μm in Giemsa-stained smears. On one previous occasion when experimental transmission to *H. zea* was achieved, only immature stages of the *Helicosporidium* were found, and a complete comparison with the isolates from the water samples was not possible. *Helicosporidium* spores were not identified in the water samples.

Other pathogens: In addition to these protozoa, a fungus (*Metarrhyzium* sp.) infected 5% of the *H. zea* larvae from 50% of the water samples. A cytoplasmic polyhedrosis virus (CPV) infected from 13 to 47% of the *H. zea* in 33% of the water samples. Cytoplasmic polyhedrosis virus-like crystals were also observed in adult *An. quadrimaculatus* from larvae exposed to water samples collected in April and May 1984 and in *Cx. salinarius* exposed as larvae to water samples collected in March, April, and May 1984.

Except for the *Telomyxa* sp. from helodid larvae, spores derived from infected organisms collected with the water samples failed to infect the target hosts.

DISCUSSION

The isolation of microsporidia spores and other pathogens from these water samples demonstrated some interesting phenomena. Frequently, microsporidia spores could be isolated from water when hosts with these spores could not be found. Also, as evidenced by *Vairimorpha* spp. and perhaps the *Pleistophora* sp., the spores in the water samples were not limited to those originating from aquatic organisms. Some spores, such as *Vairimorpha* spp. and perhaps the *Pleistophora* spp., obviously came from Lepidoptera either in the canopy above the water or the vegetation at the edge of the ditch.

The developmental stages and spores of the *Nosema* sp. were morphologically identical at the light microscope level to *Nosema algerae* (Vavra and Undeen 1970). In December 1983 there were up to 1,000 spores/ml within the size range of this *Nosema* isolate spores in the ditch water; however, the only mosquitoes collected were uninfected *Culex* spp. Since none of the other aquatic organisms were found infected with *Nosema*, it was not possible to determine its natural host.

The use of this technique to search for pathogens of selected terrestrial and aquatic insects takes advantage of the potentially broad source of pathogens present in field water samples. This type of pathogen, infectious to mosquitoes and with a persistent infectious stage, would have the attributes necessary for consideration for biocontrol. Pathogens isolated in target mosquitoes are from a sampling of infectious stages of all pathogens in close ecological contact with mosquito larvae and not limited to the pathogens from aquatic organisms only. The *Nosema* and *Vavraia* isolated in this study had not previously been found locally any other way. Identification of the original host of the isolated pathogen may be very difficult, but would not be necessary except for those pathogens which proved worthy of consideration as mosquito biocontrol agents.

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REFERENCES CITED

Andreadis, T. G. 1983. An epizootic *Amblyospora* sp. (Microspora: Amblyosporidae) in field populations of the mosquito, *Aedes cantator*. J. Invertebr. Pathol. 42:427–430.

Andreadis, T. G. and D. W. Hall. 1979. Significance of transovarial infections of *Amblyospora* sp. (Microspora: Thelohaniidae) in relation to parasite maintenance in the mosquito *Culex salinarius*. J. Invertebr. Pathol. 34:152–157.

Hazard, E. I. and B. A. Federici. 1985. Ultrastructure and description of a new species of *Telomyxa* (Microspora: Telomyxidae) from the semiaquatic beetle, *Ora texana* Champ. (Coleoptera: Helodidae). J. Protozool. 32:189–194.

Hazard, E. I. and J. Weiser. 1968. Spores of *Thelohania* in adult female *Anopheles*: development and transovarial transmission, and redescriptions of *T. legeri* Hesse and *T. obesa* Kudo. J. Protozool. 15:817–823.

Sprague, V. 1977. Zoological distribution of the Microsporidia. *In*: L. A. Bulla and T. C. Cheng, (eds.), Comparative pathobiology, vol. 2. Plenum Press. New York.

Sweeney, A. W., E. I. Hazard and M. F. Graham. 1985. Intermediate host for an *Amblyospora* sp. (Microspora) infecting the mosquito, *Culex annulirostris*. J. Invertebr. Pathol. 46:98–102.

Vavra, J. and Undeen, A. H. 1970. *Nosema algerae* n. sp. (Cnidospora: Microsporida): A pathogen in a laboratory colony of *Anopheles stephensi* Liston (Diptera: Culicidae). J. Protozool. 17:240–249.