LABORATORY STUDIES ON A NEW FUNGAL PATHOGEN OF MOSQUITOES

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INTRODUCTION. In the initial phase of studies on pathogens of insect vectors, the basic biology of the pathogen and its interaction with its host are of primary importance. Factors such as its infectivity, portal of entry, tissues or organs primarily attacked, portal of exit, and mode of infection are basic to an understanding of the parasite-host relationship. In addition to basic biological studies, methods for production and storage of the infective material must be developed, the effects of varying environmental conditions must be determined, and infectivity and virulence for appropriate mosquito species must be assessed before the pathogen is selected as a candidate for field experiments.

This paper reports the initial studies in the evaluation of *Lagenidium culicidum* (Umphlett and Huang, 1970) as a potential biological control agent of mosquitoes. Specifically, these studies pertain to (a) the life cycle of the fungus in its mosquito host; (b) the species of mosquitoes susceptible to infection; (c) effects of fungal growth in various host species; (d) mosquito susceptibility at various stages; and (e) development of a practical method for laboratory propagation and storage of infective, pathogenic inoculum.

MATERIALS AND METHODS. I. SOURCE OF PATHOGEN. In May 1969, C. J. Umphlett and C. S. Huang isolated a fungus from infected *Culex* larvae collected in Orange County, North Carolina. A pure culture was established in vitro and preliminary tests (Umphlett and Huang, 1970) with *Culex restuans* showed that the organism successfully invaded and killed the larvae. They identified the fungus as a new species of *Lagenidium* and provisionally named it *L. culicidum*. A vegetative culture from a single sporangium on agar was brought to the Technical Development Laboratories at Savannah in October 1969 for screening with several species of mosquitoes, and evaluation as a potential biological control agent.

The initial laboratory-infected larvae used in these studies were obtained by placing hemp seeds that had been on the surface of the fungal culture for 7 days in 100 ml. of distilled water with 100 second and third stage *Aedes aegypti* and *Culex pipiens quinquefasciatus*. Those larvae that became infected and died were used as infective inoculum and all tests conducted throughout the study originated with this material.

II. ROUTINE LABORATORY PRODUCTION.

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The method developed and used throughout these studies consisted of placing four infected larvae, dead less than 24 hours, in a beaker containing 100 ml. of unchlorinated tap water. Twenty-four hours later 50 mg. of Purina [3] laboratory chow, ground to pass through a 40-mesh sieve, and 25 three-day-old test larvae were added. All cultures were kept in a room maintained at 80° F and 75 percent relative humidity. The larvae became infected and usually died 72 hours later. These infected cadavers were then used for the next serial passage.

III. GENERAL TEST CONDITIONS. All tests were conducted in plastic beakers containing 100 ml. of tap water, 25 three-day-old test larvae and 50 mg. of chow in each of eight containers. Four infected cadavers of *C. p. quinquefasciatus* were then added to four containers and the remaining four were used as controls. The tests were conducted in a room maintained at 80° F and 75 percent relative humidity, with a normal daylight cycle. All of the test specimens used, except for *Aedes sollicitans,* were from colonies maintained at our insectary.

SPECIFIC TESTS AND RESULTS. I. LIFE CYCLE. There appear to be two distinct portals of entry for the biflagellate, motile zoospores; the mouth and direct penetration of the exoskeleton. Microscopic observations of hundreds of test larvae exposed to the fungal inoculum revealed that mycelial growth most frequently occurred first in the head and grew toward the posterior, infecting the anal segment and gills last. Also, mycelial growth especially in the early stages, was most abundant in the head and neck. These observations strongly suggest that the motile zoospores are normally ingested and probably enter the tissues of the host larvae through the anterior portion of the digestive tract.

In about 2 percent of the specimens observed, however, mycelial growth first appeared in the abdomen, anal segment or gills. In these larvae, with fungal growth only in the posterior portion of the body, the hyphae appeared to grow from darkly pigmented spots on the chitinous exoskeleton. These pigmented spots, two of which are visible with attendant hyphae in Figure 1, are presumed to represent the sites of zoospore penetration or attachment.

Hyphae (Fig. 2) were usually first observed about 36 to 40 hours after placing the test larvae with the inoculum. The hyphae appeared to grow via the haemocoele and had usually spread to the anal segment and gills by the end of 40 to 46 hours. The hyphae were, by that time, spread throughout the body, and about 18 to 24 hours later formed the reproductive sporangia (Fig. 3). Sporangial formation and larval death were usually simultaneous. By the next day the motile zoospores were being produced by the infected cadavers and the material was used as infective inoculum.

II. SUSCEPTIBLE MOSQUITO SPECIES. Larvae of *Ae. aegypti, Ae. triseriatus, Ae. mediovittatus, Ae. taeniorhynchus, Ae. sollicitans,*[4] *C. p. quinquefasciatus, C. fatigans, C. tarsalis,*[5] *C. nigripalpus, Anopheles albimanus, A. quadrimaculatus, A. stephensi* and *A. sundaicus* were used as the candidate species. All tests were repeated at least three times.

Except for the anophelines, all species became infected by the fungus, *L. culicidum* (Table 1). In the initial tests with *Ae. taeniorhynchus,* infection and subsequent mortalities were erratic. Close observation of the *Ae. taeniorhynchus* revealed that the test larvae began eating the cadavers used as inoculum before the fungal sporangia had an opportunity to develop the infective zoospores. The test procedure was then modified so that the

[3] Use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service or the U. S. Department of Health, Education, and Welfare.

[4] Courtesy of Oscar Fultz, Chatham County Mosquito Control Commission, Savannah, Georgia.

[5] Courtesy of R. O. Hayes, EIP, Fort Collins, Colorado.

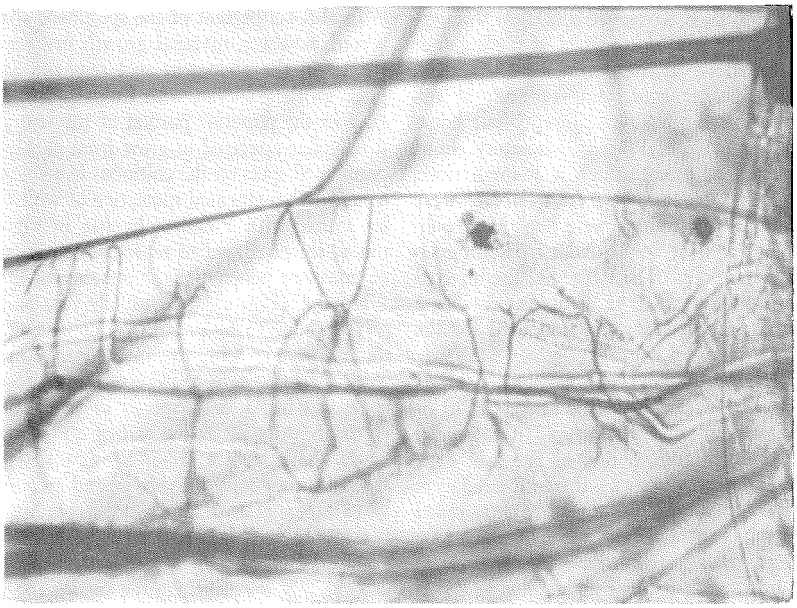

FIG. 1.—Hyphae of *Lagenidium culicidum* in anal gill of living *Culex pipiens quinquefasciatus* showing two darkly pigmented spots presumed to be sites of infection.

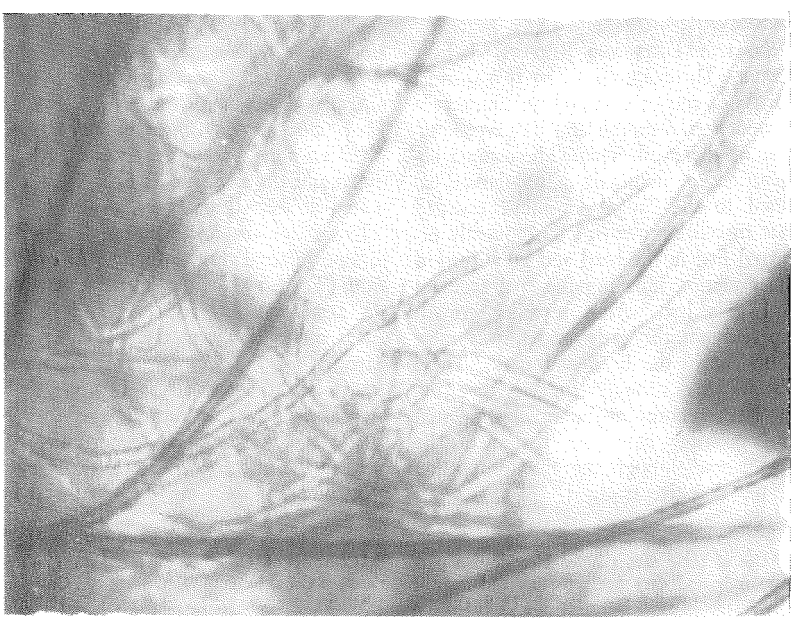

FIG. 2.—Hyphae of *Lagenidium culicidum* in head of living *Culex pipiens quinquefasciatus*.

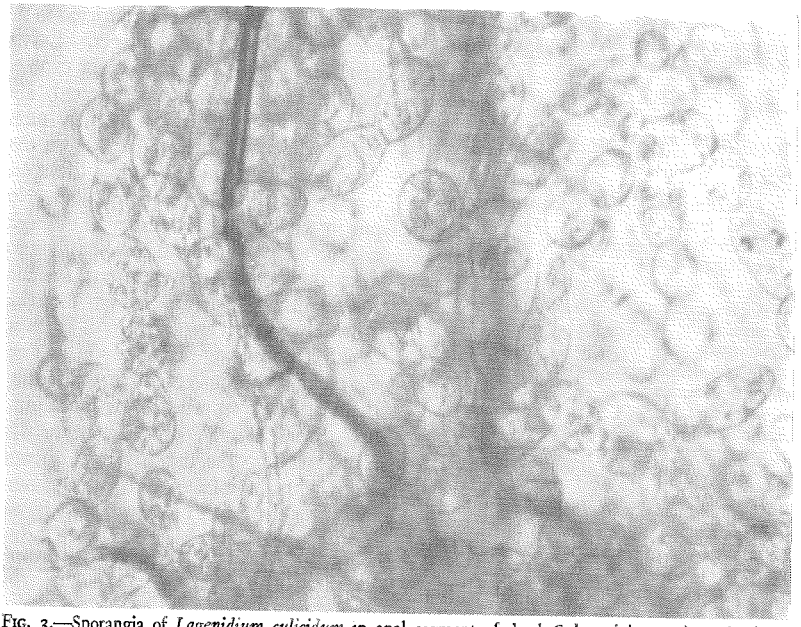

FIG. 3.—Sporangia of *Lagenidium culicidum* in anal segment of dead *Culex pipiens quinquefasciatus*.

inoculum was placed in the test container for 24 hours before the test larvae were added. This enabled the fungus to develop and release the zoospores into the water prior to the introduction of the larvae. The *Ae. taeniorhynchus* then became infected and died in all tests.

A portion of the larvae of all species was microscopically examined throughout the course of the tests. All were infected and fungal development was the same in all of the susceptible species and mortality occurred within approximately the same period of time. No difference was noted in the appearance of the hyphae or sporangia. The fungus completed its development and produced motile zoospores in all infected specimens.

III. INTERSPECIES INFECTIVITY AND PATHOGENICITY. Although all of the culicine species tested became infected with zoospores produced from the growth of *L. culicidum* in *C. p. quinquefasciatus*, there was a possibility that fungal growth in one species might so alter the physiology of the fungus that it would lose its infectivity and/or pathogenicity for other species. Using *Ae. aegypti, Ae. taeniorhynchus, Ae. triseriatus, C. p. quinquefasciatus* and *C. fatigans* as host species, the fungus was cultured in each species through three passages. Using the standard test procedures, 3-day-old larvae of each species were combined with infected cadavers of each of the other species. Microscopic observations throughout the tests revealed that all larvae became infected and in all species the fungal development was essentially identical and all larvae died approximately 72 hours after they were placed with the inoculum.

IV. HOST AGE. In the early phase of the work and before standard test procedures had been devised, infection and mortality were erratic. Careful study revealed that in those instances in which the larvae pupated and emerged as healthy adults, they usually pupated within 24

TABLE 1.—Percent mortality of larvae [a] 3 days old when exposed to *Lagenidium culicidum*.

Test species	Control [b] mortality	Days after exposure [c] 2	3	4
Aedes aegypti	0	15	100	100
Ae. triseriatus	0	0	97	100
Ae. mediovittatus	<1	13	100	100
Ae. taeniorhynchus	0	0	95	100
Ae. sollicitans	0	0	94	100
Culex fatigans	0	11	100	100
C. p. quinquefasciatus	0	12	91	100
C. tarsalis	<1	0	96	100
C. nigripalpus	0	9	97	100
Anopheles albimanus	3	0	0	2 [d]
A. quadrimaculatus	2	0	0	1 [d]
A. stephensi	<1	0	1 [d]	1 [d]
A. sundaicus	4	0	0	3 [d]

[a] At least three tests of four replicates of 25 larvae of each species.
[b] Control mortality accumulated for 4 days.
[c] No mortality during first 24 hours.
[d] Microscopic examination negative for fungus.

to 30 hours after being placed with the infective material. This led to the study of larval host-age in relation to infection. Larvae of *Ae. aegypti* and *C. p. quinquefasciatus,* which began pupating on about the 6th day with our rearing procedures, were used as test specimens. Larvae that were 1, 2, 3, 4 and 5 days of age were set up following the standard procedures. Daily observations revealed the presence of hyphae within 24 to 30 hours in test larvae that were 1 and 2 days old when placed with the inoculum. All larvae became infected and death normally occurred 40 to 48 hours after exposure. However, in larvae that were 3 and 4 days old when placed with the inoculum, hyphae were not observed until 36 to 40 hours after exposure. All larvae became infected and death normally occurred 48 to 72 hours following exposure.

Throughout these tests, all larvae that were early third instar or younger when exposed became infected and died without pupating; some late third instars occasionally pupated, but all were infected and died; early fourth instars frequently pupated and died as pupae. Microscopic examination of dead pupae revealed abundant fungal sporangia restricted to the head and cephalic appendages. Larvae that were late fourth stage when exposed usually did not become infected and pupated and emerged as healthy, uninfected adults. Of these adults, those that were not dissected lived normal life spans when fed 5 percent sucrose solution. Not one infected adult has been found throughout the entire study, and those adults produced by larvae that were exposed as late fourth instar failed to induce infection when placed with test larvae. All attempts to infect pupae were unsuccessful as were all attempts to infect adults by feeding them aqueous suspensions of inoculum.

V. STORAGE OF VIABLE INOCULUM. In routine culture the test larvae were combined with the infected cadavers 24 hours following the death of the infected larvae because the motile zoospores were being produced at that time. In order to facilitate the scheduling of laboratory tests and accumulate large quantities of viable material for projected field studies, series of tests were initiated to determine how infective material could be stored.

In those tests in which the dead larvae were held in the rearing medium at 80° F and some cadavers removed daily and used as inoculum, the material remained infective for about 5 days, but the results were erratic (Table 2). Cadavers that were placed on filter paper the day after their death and dried, produced no subsequent infection, nor did such cadavers when placed on moist filter paper and stored in sealed vials. However, cadavers that were removed from the rearing media the day the larvae died, placed in fresh tap water, and stored immediately at 60° F (before the sporangia began forming zoospores), remained infective for 2 weeks (Table 3). In test larvae infected with this material, the fungal hyphae appeared to develop normally and the larvae died on schedule, *i.e.,* 72 hours later.

DISCUSSION. In 1967 when CDC initiated a field and laboratory study of the protozoan parasite *Lankesteria culicis* (Ross) as a potential biological control

TABLE 2.—Percent infection and mortality of 3-day-old *Culex pipiens quinquefasciatus* larvae 4 days after exposure to infected cadavers that had been held in water for 1 through 7 days at 80° F.

Days Held	T-1		T-2		T-3		T-4		Controls	
	Inf.	Dead	Inf.	Dead	Inf.	Dead	Inf.	Dead	Inf.	Dead
1	100	100	100	100	100	100	100	100	0	0
2	100	100	100	100	100	100	100	100	0	0
3	100	100	98	98	100	100	100	100	0	0
4	98	98	74	74	97	97	21	21	0	0
5	25	25	32	32	11	11	16	16	0	0
6	0	0	0	0	0	0	<1	<1	0	0
7	0	0	0	0	0	0	0	0	0	0

Each figure represents the mean of four tests.

organism for *Ae. aegypti,* all of the field teams were to examine their field-collected larvae for *L. culicis* and other microorganisms that might be present (Gentile *et al.,* 1971). Several organisms other than *L. culicis* were reported from most of the survey sites, but the only fungus reported was from Clermont, Florida (Stapp and Casten, 1971). This material had growth characteristics like those of *Lagenidium,* but was in preserved specimens and was unsuitable for culture.

In the fall of 1967, Mr. Harold Bond of the Technical Development Laboratories field station in Meridian, Mississippi, collected several *Orthopodomyia signifera* larvae infected with a fungus which he tentatively identified as a member of the genus *Lagenidium,* but was unable to find a species description. Several dead, infected larvae were forwarded to the facilities in Savannah, but failed to induce infections in the laboratory. These two instances, and one of *Lagenidium giganteum* Couch as a weak parasite of mosquito larvae (Couch, 1935), are apparently the only reports of *Lagenidium* as pathogens of mosquito larvae.

This new species, *L. culicidum,* is an excellent pathogen of mosquito larvae. In the laboratory it readily infects healthy larvae of every culicine mosquito tested. In our routine laboratory production when using the proper age larvae and inoculum,

TABLE 3.—Percent infection and mortality of 3-day-old *Culex pipiens quinquefasciatus* larvae 4 days after exposure to infected cadavers that had been held in water for 1 through 14 days at 60° F.

Days Held	T-1		T-2		T-3		T-4		Controls	
	Inf.	Dead	Inf.	Dead	Inf.	Dead	Inf.	Dead	Inf.	Dead
1	100	100	100	100	100	100	100	100	0	0
2	100	100	100	100	100	100	100	100	0	0
3	100	100	100	100	100	100	100	100	0	0
4	100	100	100	100	100	100	100	100	0	0
5	100	100	100	100	100	100	100	100	0	0
6	100	100	100	100	100	100	100	100	0	0
7	100	100	100	100	99	99	100	100	0	0
8	98	98	100	100	100	100	94	94	0	0
9	100	100	100	100	100	100	100	100	0	0
10	100	100	99	99	100	100	100	100	0	0
11	100	100	100	100	100	100	100	100	0	0
12	89	89	100	100	95	95	91	91	0	0
13	76	76	83	83	51	51	92	92	0	0
14	17	17	14	14	26	26	18	18	0	0

Each figure represents the mean of four tests.

all larvae become infected and all larvae die. Not one infected larva has survived to become an adult. The production in the laboratory is a simple and straightforward procedure. No differences have been detected in any of the susceptible mosquito species in relation to pathogenicity, sporangial formation and zoospore production. Zoospores from each susceptible species appear equally infective and pathogenic for all other susceptible species. The fungus readily infects all stages of larval development except fourth instar. The data from these initial investigations indicate that *L. culicidum* is an excellent candidate for further evaluation as a potential biological control tool.

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