OBSERVATIONS ON THE VERTICAL TRANSMISSION OF A NEW MICROSPORIDIAN PATHOGEN OF *AEDES AEGYPTI* FROM THAILAND[1]

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ABSTRACT. A new microsporidian pathogen of *Aedes aegypti* from Thailand was vertically transmitted within the egg. Males played little, if any, part in vertical transmission. Infected females produced only about one-third as many progeny as uninfected females. Reduced fecundity in infected females resulted both from the production of fewer eggs and from a lower percent of hatch. In the first gonotrophic cycle not all infected females produced infected progeny, but in subsequent gonotrophic cycles some, but not all, progeny of all infected females were infected.

INTRODUCTION

Microsporidia are among the most common mosquito pathogens and are of interest as potential microbial control agents (Roberts and Castillo 1980, Roberts and Strand 1977, Chapman 1974). However, only 3 presently named species can be reliably transmitted to mosquito larvae by *per os* exposure in the laboratory (Hazard and Chapman 1977). These are (nomenclature according to Sprague 1977): *Vavraia culicis* (Weiser), *Nosema algerae* Vavra and Undeen, and *Microsporidium milleri* (Hazard and Fukuda); the latter gives only low and inconsistent rates of transmission (Hazard and Fukuda 1974). Microsporidian spores, the infectious stage, can be produced only in living hosts, and impractically large concentrations apparently would be required for *per os* exposures to result in high levels of mortality among exposed larvae (Reynolds 1970, Undeen and Alger 1975, Anthony et al. 1978a, Kelly et al. 1981). It seems likely that, as suggested by Anthony et al. (1978a), the value of Microsporidia as microbial control agents for mosquitoes will be primarily through their debilitative effects on longevity and fecundity, rather than through their immediate killing power.

A recently discovered and as yet unnamed microsporidian pathogen of *Aedes aegypti* (Linn.) in Thailand is both vertically transmitted and reliably transmittable by *per os* exposure of larvae with resulting high rates of infection (Hembree 1979). Dose-mortality studies with this agent indicate that large concentrations are required to effect mortality quickly (Hembree 1982), as with *N. algerae* and *V. culicis*. Its developmental cycle is being studied, and taxonomic placement appears to be problematic and will require time for resolution (Hazard, personal communication). Therefore, our studies on vertical transmission are presented now.

METHODS AND MATERIALS

The *Ae. aegypti* used were a US strain maintained for several years at the US Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, MD. Stock colony larvae were reared in dechlorinated tap water at 26° C in plastic trays 56 x 43 x 13 cm, 2000 larvae per tray, and fed 1:1:1 mixture of yeast, liver powder and ground high

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protein content hog chow. Pupation began during the 5th day and was mostly completed in 2–3 days. Adults were maintained at 25° C and 80% relative humidity in 60 cm^3 screened cages. Guinea pigs were provided 3 times weekly for blood meals. Five percent sucrose was constantly available. Eggs were collected on moist paper toweling, dried and stored at room temperature. Only eggs less than 1 month old were used in experiments. Hatching was effected by placing eggs in deoxygenated water with a small amount of food for 30 min under room conditions followed by 30 min under negative pressure of 17 psi.

The microsporidian was transported from the Department of Medical Entomology, US Army Medical Component, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, in infected eggs of a Thai strain of *Ae. aegypti*. The microsporidian proved to be equally infectious for the US strain of the host, which was subsequently used to maintain a stock culture of the pathogen. Fourth stage larvae infected by vertical transmission were easily recognized in black pans by white spots on the abdomen and thorax caused by large cysts of pathogen spores. These larvae were triturated in Ten Broeck type tissue grinders. The spores were quantified using a hemocytometer slide. Groups of 100 24 hr old larvae were exposed for 24 hr in 20 ml total suspension of 1000 spores/ml in 50 ml glass beakers. A small amount of food was provided during exposure. Exposure was terminated by pouring the larvae onto an organdy cloth screen and gently rinsing them with tap water to separate spores. Exposed larvae were reared in small enameled pans, 100 larvae/pan, in 1 liter of water. Pupae were collected and caged and adults were maintained as above. Experimental exposures were by these methods unless otherwise stated.

To determine presence of infection, larvae were placed on microscope slides and crushed and smeared with the tips of wooden applicator sticks. Five discrete smears, 1 cm in diam, could be placed on each slide. The smears were fixed in methanol and stained in 2% Giemsa stain in 0.01 M phosphate buffer, pH 7.4, for 1 hr. Cover slips were affixed with synthetic mounting medium. It was empirically determined that virtually all 4 day or older *per os* or vertically acquired infections would be detected by examining these smears at 650X magnification. Many infections of this age could be detected in a single microscope field. Before designating a smear as negative, adjacent fields were read across the entire smear. Percents of infection in experimental groups were determined by examining smears of at least 25 individuals if this number was available. Unexposed controls were routinely used in experiments to confirm the absence of prior infections in the experimental groups.

To determine if vertical transmission was effected by spores carried on the outside of the eggs (transovum) or by stages of the pathogen within the egg (transovarial), an egg paper from the infected colony was cut into 6 approximately equal parts. One part was hatched by routine methods. The remaining 5 parts were placed in a 5% solution of commercial sodium hypochlorite (chlorox®) from 1 to 5 min, respectively. These were then thoroughly rinsed and hatched by routine methods. Randomly selected groups of the resulting larvae were reared to the 4th stage, and the percent of vertically acquired infection in each group was determined.

To determine if males functioned in vertical transmission of the pathogen, advantage was taken of the inability of males to mate immediately after emergence, due to the necessity that their terminalia rotate 180° after emergence (Christophers 1960). About 4,000 larvae were hatched and divided into 2 approximately equal groups. One group was exposed to 1000 microsporidian spores/ml as described above. When the exposed larvae were in the 4th stage, 25 larvae were smeared to determine the percent infection. Pupae were collected and placed in separate cages for exposed and unex-

posed groups. At intervals of 4 hr for 72 hr after the first adults emerged, the adults were immobilized in a cold room and separated into 4 groups of unmated adults: exposed males and females and unexposed males and females. All adults thus separated during the emergence period were then cold-immobilized and recombined into the following combinations: (1) unexposed males × unexposed females, (2) unexposed males × exposed females, (3) exposed males × unexposed females, and (4) exposed males × exposed females. These were allowed to mate, blood meals were provided and eggs were collected for 10 days. The eggs were allowed to embryonate for 7 days before hatching. Larvae were reared to the 4th stage and 50 were smeared from each group to determine percent infection.

To determine if *per os* acquired infections were transmitted to generations subsequent to the F_1, 2000 larvae were exposed to 1000 spores/ml as described above. Twenty-five of these were smeared in the 4th stage to determine percent of *per os* acquired infections. The remainder were reared to the adult stage and allowed to produce eggs from the first gonotrophic cycle. After a 1 wk embryonation period, these eggs were hatched, the larvae reared and a random sample of 50 was smeared to determine the percent of vertically acquired infection in the F_1 generation. The cycle was repeated to produce the F_2 generation from the first gonotrophic cycle of the F_1 generation, and 100 F_2 larvae were smeared to determine the percent infection.

To determine the proportion of *per os* infected females that effected vertical transmission, the percent of infection among these progeny, and the relative numbers of progeny produced by transmitting and nontransmitting infected females and uninfected females held under the same conditions, the following experiment was conducted. Several thousand *Ae. aegypti* larvae were hatched and divided into 2 approximately equal groups. One group was exposed to 1000 spores/ml as described above. Both exposed and unexposed groups were reared to the adult stage, accumulated in separate cages and allowed to mate. Twenty-five 4th stage exposed larvae were smeared to determine percent infection. A blood meal from a guinea pig was provided.

Twenty-four hours later, the cages were placed in a cold room to immobilize the adults, and fully engorged females were selected and placed in individual plastic vials, 8 × 4.5 cm diam., with screened tops. A moist cotton pad was provided as an oviposition substrate, a piece of applicator stick was provided as a perch and a boiled raisin was kept on the screened top and replaced daily as a source of nutrient and moisture. Individual females were removed from the vials after ovipositing and smeared to confirm presence of infection. Females not ovipositing within 5 days were discarded. Eggs were collected from 100 exposed and 100 unexposed females. These were allowed to embryonate 7 days and were then hatched. The larvae from each female were reared in separate containers to the 4th stage when they were counted. All or up to 25 larvae from each exposed female were smeared to determine percent infection.

To determine if the number of eggs produced and the percent of hatch of eggs from infected females was different from that of uninfected females, the following experiment was conducted. *Aedes aegypti* eggs were hatched to produce about 2000 larvae, which were divided into 2 approximately equal groups. One group was exposed to 1000 microsporidian spores/ml as described above. All were reared to the adult stage, allowed to mate and provided a blood meal. Twenty-five exposed larvae were smeared in the 4th stage to determine percent infection. After blood feeding, exposed and unexposed females were isolated in vials and maintained as above. Eggs were collected on moist paper toweling from 25 unexposed females and from 20 exposed females. After a 7 day

embryonation period, the eggs were counted and hatched and the percent of hatch occurring in each egg batch was determined.

To determine if the number of progeny produced and the percent of vertically acquired infection among these changed in successive gonotrophic cycles in females infected by *per os* exposure as larvae, the following experiment was conducted. One thousand larvae were exposed to 1000 microsporidian spores/ml. These were reared to the adult stage, and 25 4th stage larvae were smeared to determine the percent infection. The adults were allowed to mate and were blood-fed. Ninety blood-fed females were isolated as above and allowed to oviposit. After ovipositing, each surviving female was offered another blood meal and re-isolated to produce a second egg batch. This was repeated to acquire a third egg batch from surviving females. Eggs were allowed to embryonate for 7 days and were then hatched and reared in separate containers for each egg batch. The progeny were counted in the 4th stage and up to 25 larvae from each egg batch were smeared to determine percent infection.

RESULTS AND DISCUSSION

Routine examination of smears from control groups confirmed the absence of microsporidian infection in the stock colony of *Ae. aegypti*. Thus, percents of infection given below are presumed to have resulted from either intentional *per os* exposures or vertical transmission as specified. *Per os* exposure of 24 hr old larvae for 24 hrs to 1000 microsporidian spores/ml produced 100% infection in all cases (n = 25).

That vertical transmission of the microsporidian was transovarial (within the egg) rather than transovum was indicated by the absence of a significantly reduced percent of infection in larvae hatched from ova treated for up to 5 min in 5% chlorox. Percent of infection in larvae from untreated eggs and from eggs treated 1, 2, 3, 4 and 5 min were 46%, 48%, 38%, 48%, 54% and 42%, respectively. Sodium hypochlorite is commonly used to externally sterilize insects and insect eggs (Helms and Raun 1971). In preliminary studies, extreme morphological change in spores resulted from exposure to 5% chlorox for less than 1 min. Refractility was lost and the spores appeared empty, although no evidence of exsporulation was seen. The fact that infection is maintained in association with the eggs stored under room conditions for up to 3 months, although the spores do not survive drying, (Hembree, unpublished data) is a further indication that the pathogen is transmitted within the eggs.

As shown in Table 1, males play little, if any, part in the vertical transmission of this microsporidian. One hundred percent transmission resulted from *per os* exposure of the parent group. Vertical transmission occurred only in groups that were progeny of females from the exposed parent group. Transmission through exposed females was not enhanced by mating with exposed males.

Microsporidian transmission to the F_2 generation was effected. One hundred percent transmission resulted from *per os* exposure of the parent generation. An infection rate of 36% occurred among the F_1 generation and an infection rate of 4% occurred among the F_2 generation. Infections in the F_1 generation could only

Table 1. Percents of infection in progeny resulting from cross mating of male and female *Ae. aegypti* unexposed or *per os* exposed to a vertically transmittable microsporidian.

Combination	Percent infection in progeny
Unexposed males × unexposed females	0
Unexposed males × exposed females	62
Exposed males × unexposed females	0
Exposed males × exposed females	54

have resulted from vertical transmission. However, it has not been rigorously demonstrated that vertical transmission accounted for infections in the F_2 generation. There is some possibility that uninfected F_1 larvae acquired infection *per os* by consuming spores released into the rearing medium by heavily infected siblings. An effort was made to find spores in rearing medium of the F_1 generation. Although this effort was unsuccessful, the possibility of transmission by this mechanism cannot be definitely excluded.

One hundred unexposed females isolated in vials produced an average of 100.1 (S.D. = 29.1) progeny (4th stage larvae) during their first gonotrophic cycle. One hundred exposed females, confirmed infected, produced an average of 34.1 (S.D. = 21.9) progeny (4th stage larvae) during the first gonotrophic cycle. Of the infected females, 79% transmitted infection to some or all of their progeny. The mean number of progeny produced by transmitting infected females was the same as that produced by nontransmitting infected females (34.4 vs 34.0, respectively). The mean percent infection among progeny of transmitting females was 58.9%. There was no correlation between percent infection and number of progeny produced from an egg batch ($r = 0.20$). It is apparent that infected females produce fewer progeny than uninfected females held under the same conditions.

Confirmed infected females produced fewer eggs than unexposed females, and a larger percent of the eggs produced by infected females failed to hatch. Unexposed females in this experiment produced an average of 84.2 eggs of which an average of 82.9% hatched. Infected females in this experiment produced an average of 47.6 eggs of which an average of only 62.9% hatched. The possibility that mating with infected males influenced either the number of eggs produced or the percent hatch of these eggs has not been excluded.

As shown in Table 2, in sequential gonotrophic cycles in confirmed infected females the percent of infected egg batches increased to 100%. However, all progeny of all infected females were never infected. The mean number of progeny per batch tended to decrease in successive gonotrophic cycles, but this was not compared with uninfected controls held under the same conditions. There was no correlation between percent of infection among progeny and number of progeny from infected females ($r = 0.24$). Attrition of infected females from this experiment by mortality was high. There were originally 90 blood-fed infected females. Sixty lived long enough to have completed the first gonotrophic cycle, but only 48 produced eggs within 6 days of taking a blood meal. Forty-two of 48 ovipositing females survived to take a second blood meal. Of these, only 15 completed the second gonotrophic cycle. Fourteen took a third blood meal, but only 5 completed the third gonotrophic cycle.

Debilitative effects of a transovarially transmitted microsporidian on *Ae. aegypti* have been confirmed. Males play little, if any, part in the vertical transmission of the pathogen. It is questionable whether

Table 2. Changes in dynamics of vertical transmission of a microsporidian pathogen of *Ae. aegypti* in sequential gonotrophic cycles from the same parent females.

Gonotrophic cycle	No. egg batches	Percent batches infected	Mean no. progeny per batch ± S.D.	Mean % infection
1	48	92	36.4 ± 20.9	43.7
2	15	100	33.5 ± 17.7	79.7
3	5	100	15.8 ± 10.5	67.0

the debilitative effects of this pathogen on its host qualify it for serious consideration as a microbial control agent. Although major fecundity reduction was not demonstrated, the concentration used, 1000 spores/ml, was the minimal concentration required to consistently result in 100% infection rates of larvae of the age exposed. Higher concentrations have been shown to result in high levels of larval mortality in the exposed generation (Hembree, in prep.). The effect of higher doses on fecundity deserves investigation.

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