MATERIALS AND METHODS

Establishment of the Pathogen in the Laboratory

Field-collected Aedes aegypti larvae were brought to the Department of Medical Entomology, SEATO Medical Research Laboratory, for examination. The larvae were placed in a black tray, a few dozen at a time, and observed under bright light for gross signs of pathogens. Larvae with abnormal color, size, shape or behavior were separated from the remainder of the collection and individually examined in wet mounts with bright-field microscope illumination. Larvae with Helicosporidium spores were triturated in a Ten-Broeck type tissue grinder, and the triturant was used as an inoculum to infect laboratory produced A. aegypti larvae by per oral exposure. Subsequent experiments revealed that per oral exposure of 48 hr old A. aegypti larvae to a concentration of 5×10^4 Helicosporidium spores per ml, for 24 hr, consistently produced rates of infection approaching 100 percent (Hembree, personal communication). After amplification of the original inoculum by several serial passages in the laboratory, sufficient inoculum was available to expose large numbers of larvae by methods described below. This was done to produce infected larvae and spore suspension used in the experiments described here.

Production of Infectious Material

Spore suspension used as inoculum was prepared by placing infected mosquito larvae on a net and washing them in a gentle stream of tap water. Then, they were placed in chilled phosphate buffer, 0.01 M, pH 7.6, and triturated in a tissue grinder. The spore suspension thus acquired was washed three times by centrifugation at 750 x g for 20 min at 4°C. The supernatant fraction was discarded. The precipitate was resuspended in chilled phosphate buffer by vortexing. Spore concentrations were determined by hemocytometer counts of appropriate dilutions and adjusted to 2×10^6 spores/ml (Cantwell, 1970).

Aedes aegypti eggs were hatched within a period of 1 hr as follows. The eggs were placed in deoxygenated water containing as small quantity of ground laboratory animal food, for one-half hour. They were then subjected to negative pressure of 20 psi for one-half hour. High percentages of hatch were achieved. The newly hatched larvae were placed in tap water in an enamel pan 35 x 30 x 4.3 cm and maintained for 48 hr. Care was taken that they be adequately fed and not overcrowded.

To produce infectious material for experiments, 200-48 hr old larvae were placed in each of 135 petri dishes in a volume of 19 ml tap water. One milliliter of Helicosporidium spore suspension containing 2×10^6 spores/ml, prepared as described above, was placed in each dish. Final resulting concentrations were 10 larvae/ml and 1×10^5 spores/ml. After 24 hr exposure, the

contents of each dish were transferred to one rearing pan. The larvae were fed sterilized laboratory animal food that was ground-up sufficiently to pass through a 60 mesh screen. They were reared at ambient temperature not exceeding 27°C until pupation began. These methods were known to produce infection rates of 100 percent. Two groups of 27,000 (200 larvae/pan x 135 pans) A. aegypti larvae were exposed and reared by these methods, at an interval of two weeks. Intact infected larvae from the first group and spores concentrated from the second group were preserved and stored as described below.

Preservation and Storage of Intact Infected Larvae

In Table 1 is shown the methods by which intact infected larvae and spore suspensions, produced as described above, were preserved and stored. For preservation, about 350 intact infected larvae were placed in each of 20 screw cap vials and 40 lyophilization ampoules. As much water as possible was removed with a fine tipped pipette, and the specimens were cooled in crushed wet ice for 1 hr. The twenty vials containing intact infected larvae were preserved in liquid nitrogen (Ott and Horton, 1971). This was done by first suspending the ice cold vials 2 inches over liquid nitrogen for 5 min (at about -179°C) and then lowering them into the liquid nitrogen (-196°C). After remaining in liquid nitrogen over night, one-half of the vials (10) were removed and stored in a REVCO freezer at -70°C (Class III). The remainder were stored in liquid nitrogen at -196°C (Class I).

One-half of the lyophilization ampoules containing intact infected larvae were forzen by plunging them directly into a dry-ice and acetone bath (-80°C) for 30 min. Then, the ampoules were attached to a Virtis lyophilizer and lyophilized for 24 hr (0.5 mm Hg, -60°C condenser temperature). Following lyophilization, the ampoules were sealed with a torch. Half (10) of them were stored in a REVCO freezer at -70°C (Class V), and half (10) of them were stored in the dark at room temperature of about 25°C (Class X).

The remaining 20 lyophilization ampoules containing intact infected larvae were vacuum dried by placing them directly on a Virtis lyophilizer without freezing them in dry-ice and acetone. After 24 hr on the lyophilizer, the ampoules were sealed with a torch. Ten of these ampoules were stored in a REVCO freezer at -70°C (Class IV), and 10 of them were stored at room temperature in the dark (Class IX).

Preservation and Storage of Spore Suspension

Spore suspension was produced as described above. After washing the spores two times, the final centrifugation pellet was kept in the refrigerator over night. Then half of the spores were suspended in Extender 1 and half of them in Extender 2 (Appendix). The extenders were chilled to avoid cold shock (First, 1971).

For freezing, 35 ml of extender was added to 5 ml spore suspension. Subsamples (4 ml) of this solution were transferred

into each of 10 screw cap vials. After freezing in liquid nitrogen, the specimens were stored in a liquid nitrogen refrigerator (Class II-1 and II-2 for Extender 1 and 2, respectively) and in a REVCO freezer (Class VI and VII for Extender 1 and 2, respectively).

For lyophilization, 5 ml spore suspension was mixed with 10 ml Extender 2. Subsamples (1.5 ml) of this were transferred to each of 10, 2-ml ampoule for lyophilization. Lyophilized material was kept in the dark at a room temperature of about 25 ℃ (Class XI).

Determination of Effect of Preservation and Storage on Infectivity

To determine the effects of the various preservation and storage methods used on the infectivity of Helicosporidium spores, it was necessary to have a standard for comparisons. A dose-response curve using fresh spores as inoculum for per oral exposure was established, using the same methods subsequently to be used to assay the stored material, with the exception that 10 points were determined on the standard curve, where as three or four were routinely sought for the assay of stored material.

Two groups of 48 hr old A. aegypti larvae, produced as described above, were exposed to each of 10 doses of spores. The doses used were: 2×10^3 ; 1×10^4 ; 2×10^4 ; 3×10^4 ; 4×10^4 5×10^4 ; 6×10^4 ; 7×10^4 ; 8×10^4 ; 1×10^5 spores/ml. Two-hundred larvae were placed in each exposure container (plastic petri dishes), containing a total volume of 20 ml spore

suspension. Exposures were of 24 hr duration at 25°C. Subsequent to exposure, the larvae were placed on a fine mesh cloth screen and rinsed in a gentle stream of tap water to separate them from the spore suspension. The larvae from each exposure container were placed in an appropriately labeled, sterilized rearing pan and reared as described above. When pupation began, usually 5-6 days subsequent to exposure, a sample of 50 larvae were randomly selected to represent each spore dose tested. These were smeared with wooden applicator sticks onto glass microscope slides, five discrete smears per slide and fixed with methanol for 1 min. After fixing, the smears were stained for 1 hr in 3 percent giemsa stain, washed briefly in tap water and differentiated for 3 min in Tris buffer, 0.01 M, pH 7.6. The slides were air dried and coverslides were applied. The stained preparations were examined microscopically with bright field illumination. They were screened at 430 magnification to detect conspicuous infections. Less apparent infections were searched for at 1000 x oil immersion. Percentage of transmission at each dose rate was determined. These data were plotted against log dose-rate on semi-log graph paper to produce a dose-response curve. A concentration that theoretically would infect 50 percent of larvae exposed under the defined conditions was estimated from the curve (IC_{50}).

In this and all subsequent experiments, control groups were reared in parallel with experimental groups to confirm the absence of endogenous pathogens in the stock A. aegypti colony. Mortality during rearing of both control groups and experimental

groups was recorded daily.

Determination of the effects of preservation and storage proceeded in two stages. Four weeks after storage, a preliminary determination of effects was made. The objectives of this preliminary evaluation were to determine if transmission with stored material could be achieved at all and to determine a dose range within which transmission would be greater than zero but less than 100 percent, so that a dose-response curve could be constructed using doses within that range. Preliminary evaluations were made in all classes at the following doses: 5×10^2 ; 5×10^3 ; 5×10^4 ; 5×10^5 spores/ml. These dosages were purposefully chosen to cover a wide range to meet the objectives of the preliminary test, although they might have resulted in fewer points being available for the construction of dose-response curves from which IC_{50}'s could be estimated. After the preliminary evaluation, definitive evaluations were made at 8, 12 and 16 weeks after storage, and dose-response curves were constructed from which IC_{50}'s could be estimated for comparison with the IC_{50} determined from the standard curve. Definitive tests were purposefully made within a narrower range of dosages with the intent that three or four points would be acquired for the more accurate construction of the dose-response curves.

Attention is here invited to the timing of experiments. Because of limited insectary space, it was not impossible to produce all material for subsequent treatment and evaluation at the same time. Therefore, infected larvae to be stored intact were

produced initially. Two weeks later infected larvae from which spores were recovered to be stored as spore suspensions were produced. Subsequent experiments likewise were conducted at two week intervals. After each storage interval, material stored as intact infected larvae was evaluated, and two weeks later material stored for the same length of time as spore suspension was evaluated.

Material that was stored either in liquid nitrogen or in the REVCO freezer was retrieved and allowed to thaw at 4°C. Lyophilized and vacuum dried materials were reconstituted with water. Intact infected larvae were triturated with a Ten-Broeck type tissue grinder, and the triturant was washed twice as described above. Spore suspensions were washed twice to separate them from the extender. Quantification of spore numbers was made with a hemocytometer. Appropriate dilutions of spores were made (depending on results of preliminary evaluation or previous definitive evaluations), and 48 hr old A. aegypti larvae were exposed to them and subsequently reared as described above. When pupae began to appear, randomly selected representative samples of 50 larvae from each dose of each class tested were collected, processed and examined as described above to determine percents of transmission. These were graphed and IC_{50}'s estimated as described above. These were compared with the standard curve and IC_{50} to assess the effects of the preservation and storage methods and the duration of storage on the infectivity of Helicosporidium spores. Mortality of both experimental and control groups was recorded daily.