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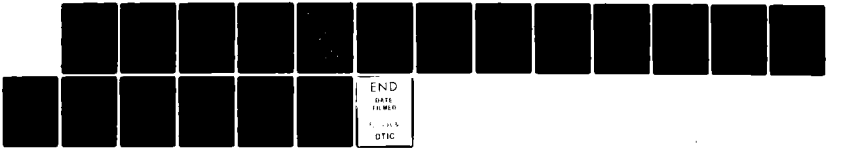

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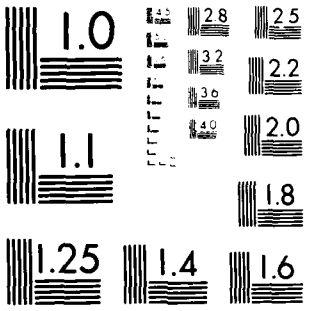

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The first enzyme-linked immunosorbent assay for a microsporidian is described. The assay detects as little as 2 ng of spore homogenate protein and as few as 2000 intact spores. Several timesaving and reagent-conserving modifications of traditional ELISA protocols are employed.

Individuals of 103 taxa of aquatic animals were surveyed as possible intermediate hosts for Amblyospora. No successful transmissions were achieved with any of these animals.

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OFFICE OF NAVAL RESEARCH

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Task No. NR 205 - 035

Annual Technical Report Number 3

Transmission of Microsporidian Parasites of Mosquitoes

By

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The microsporidia are obligate intracellular protozoan parasites of a wide range of vertebrate and invertebrate animals. They are characterized by the formation of unicellular spores, each of which contains a uninucleate or binucleate sporoplasm and a specialized infection apparatus, the polar filament. Species of the genus Amblyospora and closely related genera are among the most common and widely distributed parasites of mosquitoes.

Microsporidia of the genus Amblyospora are transovarially (vertically) transmitted from an infected female mosquito to her progeny and exhibit dimorphic development. One developmental sequence leads to formation of diploid spores in adult female hosts and the other leads to extensive multiplication (merogony) followed by meiosis and formation of numerous haploid spores (Fig. 1) packaged in groups of eight inside pansporoblast membranes. The haploid spores are produced in fourth instar larvae, and larvae with patent infections usually do not pupate and die several days after healthy larvae have pupated. These spores are refringent and result in a milky-white coloration of the infected larvae when they are viewed against a dark background.

The developmental sequence which leads to diploid spore formation is linked to blood-feeding and ovarian development of the female host. The diploid spores are formed in response to the hormone 20-hydroxyecdysone which is released by the ovaries following a blood meal (Lord, 1982). These spores are believed to function solely in vertical transmission (Andreadis and Hall, 1979a). Therefore, the haploid spores are believed to be responsible for horizontal transmission. All attempts to infect healthy larvae with haploid spores have been unsuccessful to date (Kellen et al., 1965; Chapman, 1974). Andreadis and Hall (1979b) have suggested that the haploid spores may infect an intermediate host and undergo another cycle of development prior to becoming infective once again for the mosquito host. However, the possibility that the spore may merely require some type of conditioning prior to becoming infective has not been ruled out. The mechanism of horizontal transmission of these parasites must be elucidated before their potential for biological control of mosquitoes can be assessed.

We have shown that vertical transmission alone is not sufficient for maintenance of at least some of these parasites in nature. However, at the present time, none of these parasites have been successfully transmitted in the laboratory except by vertical transmission.

This contract is concerned with the microsporidian genera Amblyospora and Parathelohania and certain species of other genera which have dimorphic life cycles and are transovarially transmitted in mosquitoes. The primary objectives of this research are to work out the life cycles of selected parasites and to determine the mechanism of horizontal transmission of the parasites from mosquito to mosquito.

This research was supported in part by the Office of Naval Research, Microbiology Program, Naval Biology Project, under Contract #N00014-80-C-0172, NR 205 - 035.

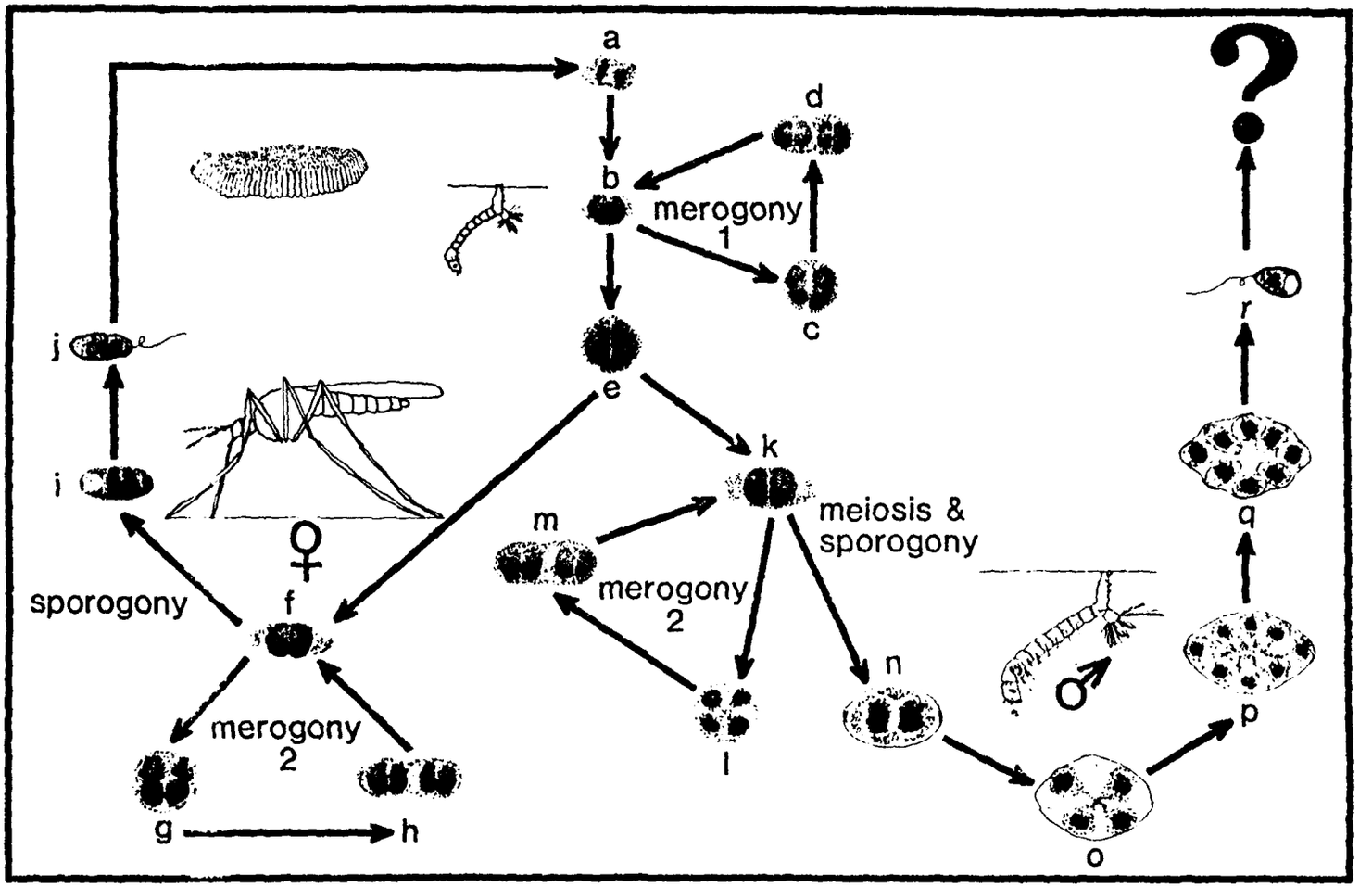

Fig. 1. Life cycle of Amblyospora sp. in Cx. salinarius. (a) Binucleate sporoplasm, (b-d) primary diplokaryon and stages of the 1st merogony in oenocytes of embryonated eggs and young larvae, (e) transitional diplokaryon, f-h) secondary diplokaryon and stages of the 2nd merogony in oenocytes of newly emerged adult females, (i) sporoblast, (j) mature spore, (k-m) secondary diplokaryon and stages of the 2nd merogony in adipose tissue of young male larvae, (n) binucleate sporont, (o) quadrinucleate sporont, (p) octonucleate sporont, (q) pansporoblast containing eight sporoblasts, (r) mature haploid spore.

Intermediate Host Studies

I. Spore Feeding Experiments

To survey possible intermediate hosts of Amblyospora sp., pairs of jars were used, an experimental jar and an assay jar, each containing about 500 ml of aged tap water. The experimental jar contained one to many individuals of a candidate intermediate host species, with a piece of aluminum screening or a thin layer of sand for those species that required a solid substrate. Predators were fed live infected C. salinarius (containing haploid spores) larvae, scavengers were fed mashed infected C. salinarius larvae and Koi Goldfish Food. The assay jar contained healthy C. salinarius larvae. Five healthy first instar larvae were added each day to each assay jar, since young larvae are probably more easily infected per os than older larvae. About 1.5 ml of water from the bottom of each experimental jar, hopefully containing some infective Amblyospora stage, were transferred daily to the appropriate paired assay jar. Both jars were gently stirred daily to suspend at least briefly any infective stages in the water column. Fourth instar C. salinarius in the assay jar were examined for the white coloration due to an overt infection of Amblyospora sp. To check for transovarial infections, adult C. salinarius were reared from the assay jars, the females fed blood and allowed to oviposit, the larvae reared until pupation began, and the second generation fourth instar larvae examined for overt infections.

A total of 142 replicate jar pairs, each containing one to many individuals of more than 103 taxa of aquatic animals, were surveyed (Table 1). These totals exclude Protozoa, Rotatoria, and Nematoda, which were present in most or all of the jars. From the assay jars, more than 1341 female and 1435 male fourth instar C. salinarius larvae were reared and examined for overt infections (sex determined at adult emergence). None of the larvae showed Amblyospora infections. From this generation of adult female C. salinarius, more than 1291 egg rafts were produced, and a second generation larvae also showed no infections.

The following should also be noted: 1) Amblyospora spores had a chance to infect the healthy C. salinarius per os in all assay jars, but did not cause infections, 2) Aging of the spores for up to 2-4 months did not produce infectivity, 3) Passage of the spores through the guts of the experimental animals, probably many times, did not produce infectivity, and 4) No infections from microsporidians present in the wild-caught hosts were detected in the assay C. salinarius larvae.

Miscellaneous experiments-- Various attempts were made to cause Amblyospora spores to germinate, including: 1) Crushing tissues of aquatic animals in a spore suspension, including ostracods, amphipods, spiders, beetle larvae, and phantom midges. 2) Feeding spores to crayfish, dragonfly larvae, damselfly larvae, water scorpions, beetles, Anopheles larvae, snails, and mosquitofish. In experiment 1, no germination was seen. In experiment 2, the spores usually did not germinate, but 7 germinated spores were found in the gut of a Pantala flavescens dragonfly larvae 2 hrs after feeding. Guts of some of the other animals had empty spore walls or dissociated polar filaments. However, no infections from any of these hosts were produced in the assay experiment, and the germination experiments were abandoned.

TABLE 1

Animals tested as intermediate hosts of Amblyospora sp. in Culex salinarius. An asterik (*) indicates a probably inadequate test. A (+) indicates many replicates or individuals. For life stages, A = Adult, I = Immature, i.e. Juvenile, Larva, Nymph.

Taxon	Number of Replicates	Number of Individuals	Life-Stage
PROTOZOA			
Mastigophora	115	+	A
Sarcodina	1	+	A
Ciliata	115	+	
PORIFERA	1	3	A
PLATYHELMINTHES			
Turbellaria	3	+	AI
ROTATORIA	+	+	AI
NEMATODA	+	+	AI
NEMATOMORPHA	1	2	A
ANNELIDA			
Oligochaeta	2	9	A
ARTHROPODA			
Crustacea			
Cladocera	2	+	A
Copepoda	3	+	AI
Ostracoda	7	+	AI
Amphipoda			
Hyalella azteca	1	+	AI
Decapoda			
Palaemonetes sp.	1	3	AI
Procambarus fallax	3	12	I
Arachnida			
Hydracarina	1	+	AI
Araneida			
Dolomedes sp.	2	2	I
Pisaurina mira*	1	1	I
Insecta			
Ephemeroptera			
Caenis sp.	1	2	I
Callibaetis sp.	1	3	I
Centroptilum hobbsi*	1	2	I
Hexagenia munda	1	2	I
Stenacron interpunctatum	2	13	I

Table 1 continued

Taxon	Number of Replicates	Number of Individuals	Life-Stage
Odonata			
Calopteryz maculata	1	+	I
Anomalagrion hastatum	1	+	I
Argia sedula	2	9	I
Enallagma cardenium	1	2	I
E. signatum	2	6	I
Ischnura posita	1	4	I
I. ramburii	3	+	I
Anax longipes	2	3	I
Epiaeschna heros	2	2	I
Nasiaeschna pentacantha	1	1	I
Gomphus minutus	1	5	I
Progomphus obscurus	1	2	I
Macromia georgina	1	1	I
Tetragoneuria sp.	1	1	I
Erythemis simplicicollis	1	11	I
Libellula sp.	1	3	I
Pachydiplax longipennis	1	7	I
Pantala flavescens	2	16	I
Perithemis tenera	1	1	I
Tramea sp.	1	4	I
Hemiptera			
*Hydrometra martini**	1	6	AI
Mesovelia mulsanti	1	9	AI
Gerris sp.*	1	3	AI
Microvelia sp.	1	10	AI
*Buenoa margaritacea**	1	1	A
Notonecta indica	2	7	AI
N. irrorata	1	1	A
Plea striola	1	4	AI
Ranatra nigra	1	7	AI
Belostoma lutarium	1	3	A
B. testaceum	3	8	AI
*Lethocerus griseus**	1	1	A
Corixidae sp.*	1	4	A
Megaloptera			
Corydalus cornutus	1	2	I
Sialis americana	1	1	I
Trichoptera			
Cheumatopsyche sp.	1	8	I
Hydropsyche sp.	1	7	I
Coleoptera			
Peltodytes oppositus	1	12	A
Acilius semisulcatum	1	4	A
Anodochilus exiguus	1	6	A
Coptotomus interrogatus	1	8	A
Desmopachria mutchleri	1	1	A
Hydroporus falli	1	3	A

Table 1 continued

Taxon	Number of Replicates	Number of Individuals	Life-Stage
Coleoptera (cont.)			
Laccophilus fasciatus	1	6	A
L. sp.	1	4	A
Dytiscidae sp.	3	5	AI
Dineutus carolinus	1	5	A
Berosus exiguus	1	10	A
B. infuscatus	1	5	A
Enochrus sublongus	2	4	A
Hydrochara sp.	1	2	A
Tropisternus lateralis	1	5	A
Hydrophilidae sp.	1	7	I
Helichus striatus	1	1	A
Dubiraphia quadrinotatus	1	3	AI
Elodes sp.	1	+	I
Diptera			
Telmatoscopus superbus	1	22	I
Psychodidae sp.	7	+	I
Aedes aegypti	1	+	I
A. triseriatus	1	+	I
Anopheles quadrimaculatus	1	+	I
Culex pilosus	1	4	I
C. territans	1	4	I
Chaoborus sp.	1	22	I
Simuliidae sp.	1	28	I
Chironomidae sp.	1	+	I
*Culicoides mississippiensis**	2	+	I
Hedriodiscus trivittatus	1	13	I
Muscidae sp.	1	9	I
MOLLUSCA			
Gastropoda			
Goniobasis sp.	1	9	A
Gyraulus sp.	2	+	AI
Physa sp.	2	+	AI
Planorbella scalare	1	+	AI
Pomacea sp.	3	+	I
Pelycypoda			
Corbicula manilensis	2	5	AI
Pleurobema pyriforme	2	5	AI
PISCES			
Gambusia affinis	3	9	AI
Chaenobryttus coronarius	1	1	I
Lepomis marginatus	1	1	I
AMPHIBIA	1	2	I
	142+	438+	

Two other experiments were attempted: 3) Rearing healthy C. salinarius larvae from egg to fourth instar larvae in water from 3 different ponds, and 4) Rearing halthy C. salinarius in water containing Amblyospora spores at 38 deg. C. No overt infections were found in full grown C. salinarius larvae in either of these experiments.

II. Serological Studies

Another approach to searching for candidate intermediate hosts is the use of specific antibodies to Amblyospora in ultrasensitive serological assays of wild collected aquatic invertebrates. Final work on an enzyme-linked immunosorbent asay (ELISA) was completed. A description of this technique follows. Infected fourth instar larvae were harvested, rinsed in tap water, macerated in a tissue grinder, and stored at -80°C in phosphate-buffered saline (PBS), pH 7.4, until sufficient number had been accumulated for efficient purification. Thawed macerates were centrifuged for 2 min at 6μ to remove large mosquito fragments. Spores were then concentrated by centrifugation for 5 min at 700g and layered onto a 0-20% Ludox HS-40 (E.I. du Pont de Nemours, Wilmington, Del.) continuous density gradient (Undeen and Alger, 1971) and centrifuged for 4 hr at 1500g at 4°C. After four washes in large volumes of PBS, pH 7.4, the spores were counted with a hemacytometer and stored at -80°C until needed.

Spore homogenates were prepared by combining thawed spore suspensions at 10^8/ml with an equal volume of 0.5-mm glass beads and homogenizing in a Braun MSK tissue homogenizer (Knell, 1975). Homogenates were checked with the hemacytometer to ensure at least 95% spore disruption. Homogenate protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.), using bovine serum albumin (BSA) (Fraction V powder, Sigma, St. Louis, Mo.) as control.

Two 25-g BALB/c mice received intraperitoneal injections of 100 μg homogenate protein each in PBS, pH 7.4, emulsified with an equal volume (0.35 ml) of Freund's complete adjuvant. At intervals of 2 to 3 weeks they received two additional injections of 100 μg homogenate protein without adjuvant. One month following the final injection they were bled from the ventral tail vein and their blood was pooled. Blood was allowed to clot for 1 hr and the serum was removed and heat inactivated (Kabat and Mayer, 1961). Immunoglobulin was precipitated with 38% ammonium sulfate, dissolved in and dialzyed three times against large volumes of PBS, pH 7.4, and stored at a 1:5 dilution at 4°C prior to use.

Indirect ELISA's (Voller et al., 1976) were performed, using sheep anti-mouse immunoglobulin, kindly furnished by G.A. Gutman, as second antibody. The sheep antibody was conjugated to alkaline phosphatase (Sigma) according to the protocol of Clark and Adams (1977).

In order to determine optimum reagent concentrations, checkboard titrations were set up using all combinations of antigen at 10, 3.3, 1.1, 0.37, and 0.12 μg/ml, mouse anti-Amblyospora immunoglobulin at 10, 3.3, 1.1, 0.37, 0.12, 0.04, 0.014, and 0.005 μg/ml, and sheep anti-mouse immunoglobulin conjugate at dilutions of 1:100, 1:500, and 1:1,000.

All assays were performed in 96-well flat-bottomed Linbro EIA plates (Flow Laboratories, McLean, Va.), using 0.1-ml quantities of reagents. Plates were coated with antigen in PBS, pH 7.4, or carbonate buffer, pH 9.6, (Clark and Adams, 1977), incubated 1 hr at 37°C, and washed four times for 3 min each with PBS-Tween (PBST) (Clark and Adams, 1977). Following this mouse anti-Amblyospora immunoglobulin was introduced in PBST with 0.05% BSA (PBST-BSA), incubated 1 hr at 37°C and unbound immunoglobulin removed by washing. Next, sheep anti-mouse immunoglobulin conjugate in PBST-BSA was introduced and the plate incubated and washed as before. Finally, substrate (p-nitrophenyl phosphate, Sigma, 1 mg/ml) in diethanolamine buffer, pH 9.8 (Clark and Adams, 1977), was added, incubated for 1 hr at room temperature, and the reaction stopped by the addition of 0.05 ml of 3 M NaOH. Six- or ten-replicate buffer and normal mouse serum (NMS) controls were run with each assay. The absorbence at 405 nm of each well was determined with a Titertek Multi-Skan 8-channel photometer (Flow Lab.).

Ten-replicate titrations of spore homogenate at 50, 30, 15, 5, and 2 ng protein per well, and of intact spores at 40,000, 20,000, 10,000, and 5,000 per well, were performed. Homogenate titrations were assayed as described above; spore titration plates were coated and incubated overnight at 4°C rather than 1 hr at 37°C.

The relationships between homogenate protein mass, Amblyospora spore number, and absorbence at 405 nm were determined by weighted regression analysis (Draper and Smith, 1981, p. 108). The sensitivities of the assays were determined by inverse regression (Draper and Smith, 1981, pp. 47-50), and expressed as the largest value of homogenate mass or spore number for which the estimated absorbence differs significantly from that of the mean of the control absorbences. This method is preferable to the usual approach of comparing just the means of the control and the lowest concentration of material tested by the t test, because it uses information from the entire titration 50 or 40 observations) rather than just those from the single lowest concentration (10 observations). Sensitivity to homogenate protein is expressed in mass rather than concentration because the photometer absorbences are directly proportional to mass of material (Titertek manual, Flow Lab).

The assay was most sensitive using mouse anti-Amblyospora immunoglobulin at 3.3 µg/ml and sheep anti-mouse immunoglobulin conjugate at 1:100 dilution. Assays using PBS and carbonate-coating buffers did not differ in sensitivity so the former was adopted for all subsequent work. Amblyospora homogenate masses greater than 50 ng/well and intact spore numbers greater than 50,000/well gave off-scale readings on the photometer.

Data from the Amblyospora homogenate and spore titrations are shown in Figures 2 and 3, respectively. The equation

$$y = 0.017x + 0.156$$

explains 97% of the total weighted variation in Figure 1, and

$$y - 27.3x + 0.062$$

explains 99% of that in Figure 3. The estimated sensitivities of these two assays are 1.57 ng homogenate protein and 1710 spores, respectively ($P<0.05$, one-sided test).

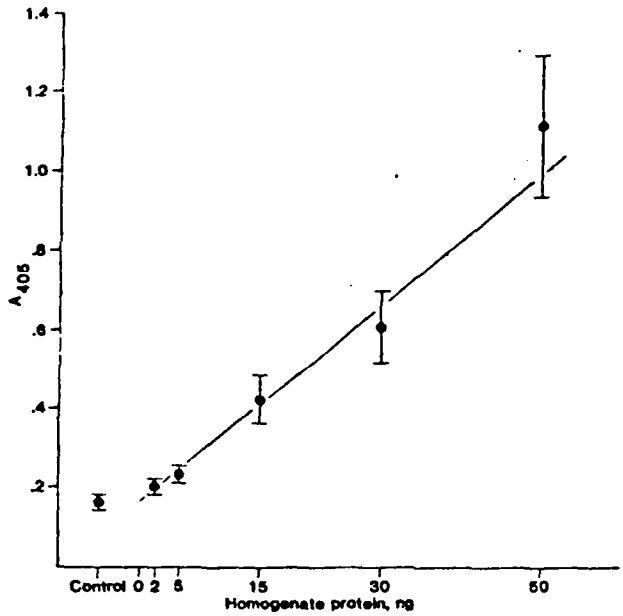

Fig. 2. The relationship between Amblyospora homogenate protein mass and absorbance at 405 nm in the indirect ELISA. Data shown are means and 95% confidence intervals for ten observations at each mass; line was fitted by weighted linear regression (Draper and Smith, 1981).

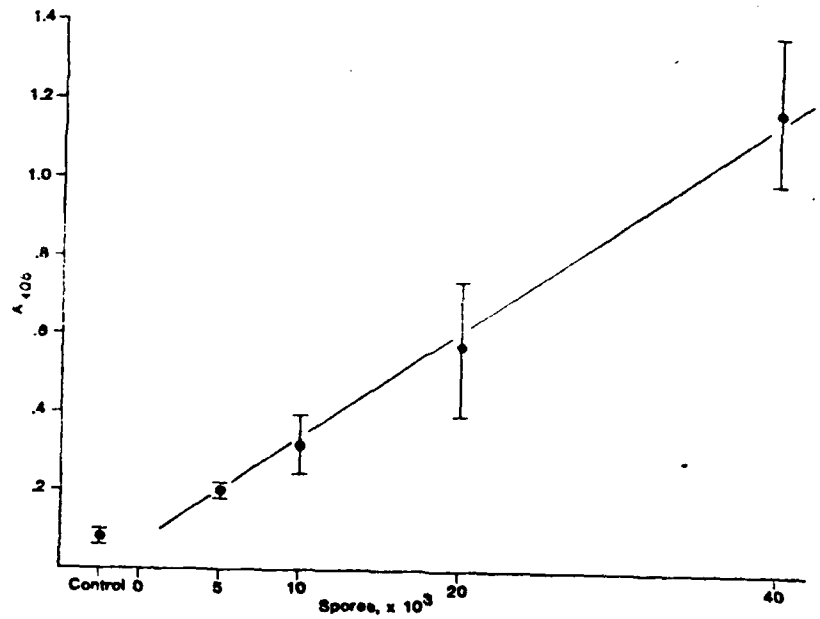

Fig. 3. The relationship between Amblyospora spore number and absorbance at 405 nm in the indirect ELISA. Data and line as in Figure 1.

The indirect rather than the more popular double antibody sandwich (Voller et al., 1976; Clark and Adams, 1977) ELISA configuration was initially chosen because the assay will eventually be performed using monoclonal antibodies (Kohler and Milstein, 1975) and the indirect configuration lends itself better to a screening assay for hybridoma antibody production. (It also explains the use of mice rather than rabbits). However in practice the indirect ELISA assay has usually proven to be about ten times more sensitive in assays for antigen (Kaupp 1980; Crook and Payne, 1980). It has the additional advantage that whereas specific antibody may be in short supply, particularly in the case of antibodies to microbial pathogens which are themselves precious, second antibodies are generally abundant, and may even be purchased commercially as enzyme conjugates. This essentially doubles the usable supply of specific antibody and also makes it possible to assay for several different antigens using specific antibodies produced in the same vertebrate species without having to make a different conjugate for each.

The procedure described here differs from traditional protocols in three other respects. First, the use of the photometer, which reads directly through the wells of the EIA plate, obviates the need to dilute and read each sample separately, speeding up the process and making it possible to reduce the reagent volume to 0.1 ml, the minimum required to coat the plate [plates with half-volume (0.05 ml) wells are also becoming available]. Second, reaction times have been drastically shortened (cf. Korpraditskul et al., 1979) so that a complete assay, including washes and reading, can be run in less than 6 hr. Since each plate has 60 usable wells (the outer wells of EIA plates tend to give discordant readings), hundreds of assays can be run in a day. Finally, PBS, pH 7.4, was substituted for carbonate, pH 9.6, as the coating buffer. This eliminates the need to make up the unstable carbonate buffer fresh each week and reduces the chances of adding a reagent in the incorrect buffer. (Crook and Payne, 1980, found that their anti-baculovirus ELISA was actually more sensitive with the PBS-coating buffer.)

This is the first ELISA reported for a microsporidian. Its sensitivity for detecting antigen protein is comparable to that of ELISA assays insect viruses (Kelly et al., 1978a, b; Kaupp 1980; Crook and Payne, 1980; Longworth and Carey, 1980). Aside from the immediate goal of identifying intermediate hosts of Amblyospora sp., assays like this one are essential for epidemiological studies of microsporidia occurring as natural epizootics or in biological contro programs. For example the ability to detect fewer than 2000 spores will make it possible to identify prepatent infections in young C. salinarius larvae.

Life Cycle Studies

The life cycles of 3 microsporidia were studied. These are: (1) a new genus and species in Aedes aegypti having developmental sequences intermediate between Amblyospora and Tuzetia, (2) Polydisprenia caecorum in Culex quinquefasciatus and Culiseta inornata and (3) Hazardia milleri in Cx. quinquefasciatus. All but one (H. milleri) were found to undergo meiosis producing haploid spores. Spores of the first species are infectious to healthy larvae producing gametes that fuse to form diplokarya in young larvae. Descriptions of these will be included in the final report.

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