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Use of Immunoassays in Haplosporidan Life Cycle Studies

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Abstract.—The development of mitigating measures for the major oyster diseases has been hindered by our poor understanding of the life cycles of the pathogens. Evidence from epidemiological studies and transmission experiments suggests that an intermediate host is present in the life cycle of *Haplosporidium* species. Immunoassay is a valuable tool for identifying parasite antigen in an intermediate host, and, because of the potential for stage-specific antigens, assays incorporating polyclonal antibodies may be more effective than assays incorporating monoclonal antibodies. Rabbit antibody against purified spores of *Haplosporidium costale* recognized spores in paraffin sections of oyster tissue, but the antibody did not recognize plasmodia of *H. costale*.

One of the major obstacles to the mitigation of parasite-induced oyster mortality is our poor understanding of the life cycles of the parasites. Four genera contain important disease agents—*Haplosporidium, Marteilia, Bonamia*, and *Perkinsus*—but only species of *Perkinsus* have life cycles that are known (Perkins and Menzel 1966). *Bonamia ostreae* may be transmissible directly (Elston et al. 1986), but the infective stage has not been identified. The lack of information on life cycles has hindered our ability to interpret field observations and has limited many lines of investigation. For example, *Haplosporidium nelsoni* (MSX) moves hundreds of kilometers up Chesapeake Bay in response to increased salinity that results from drought conditions. Because the life cycle and infective stage of *H. nelsoni* are unknown, we do not know how the parasite moves or what stage in the life cycle responds to the change in salinity. Most diseases of oysters have not been transmitted in the laboratory, so it has been impossible to investigate, under controlled conditions, infective dose, defense reactions, pathogenicity, and control measures. Although some management techniques have been developed through field manipulations of diseased oysters, sound management recommendations are also hindered by our poor understanding of the life cycles of the parasites.

A major question is whether parasite transmission between oysters is direct or via an intermediate host. Direct experimental transmission of *Haplosporidium* spp. and *Marteilia* spp. via spores has been unsuccessful and implies that an intermediate host may be involved in the life cycles of these pathogens. Andrews (1984) listed evidence for and against the existence of other hosts in the life cycle of *H. nelsoni*. Intermediate hosts should be distinguished from alternate or reservoir hosts because these terms have been used interchangeably in the literature on oyster diseases. For *H. nelsoni*, an intermediate host is a host, other than oysters, in which some development occurs that is essential for completion of the parasite's life cycle. A reservoir host is any host that serves as a source of *H. nelsoni* from which oysters can become infected. The existence of a reservoir host has been postulated for *H. nelsoni* because spores have only rarely been encountered, yet parasite prevalence is high (Farley 1967; Andrews 1984). Most other species of *Haplosporidium* and those of *Marteilia* sporulate regularly, and a reservoir host is not required to account for the observed prevalence. However, an intermediate host may be required for completion of all haplosporidan life cycles.

Life Cycle Hypotheses

All species of *Haplosporidium* produce spores, and insights into the life cycle can be gained by examining the fate of spores in life cycles of other spore-producing protozoa. Species in the phyla Myxozoa and Microspora and many species in Apicomplexa produce nonmotile spores. In the life cycles that have been elucidated for the species of these phyla, the spore is always eaten by the intermediate or final host, and the sporoplasm emerges from the spore in the gut of the new host. No life cycle is known in which the sporoplasm hatches from the spore in the external environment and exists as a free-living sporoplasm. Thus, it is plausible to hypothesize that spores of *Haplosporidium* spp. are eaten. Because all attempts to infect oysters with spores of *H. nelsoni* and *H. costale* have failed (Andrews 1984), perhaps spores are eaten by an unknown intermediate host.

Four species of *Haplosporidium* are known from the oceanic and estuarine waters of the Chesapeake Bay region. *Haplosporidium costale* in oysters sporulates in connective tissue and causes rapid death of the host. When an oyster dies and gapes, scavengers, such as crabs, amphipods, fishes, and ciliates, feed on the tissue of the oyster, and any of these scavengers could be an intermediate host. Similarly, *Haplosporidium* sp. in the mud crab *Panopeus herbsti* sporulates in all tissues of the host, and the weakened crab is probably ingested by a predator. The oyster parasite *H. nelsoni* sporulates only in digestive diverticula, and spores are probably released from live oysters after localized exfoliation of the diverticula epithelium. *Haplosporidium* sp. from the naval shipworm *Teredo navalis* sporulates in all tissues and can cause death of the host, although spores may be released from live naval shipworms during exfoliation of gill epithelium. Ingestion by scavengers seems unlikely because few organisms could enter the burrow. Spores of *Haplosporidium* spp. in oysters and the naval shipworms probably are ingested by ciliates, small crustaceans, or suspension feeders after the spores leave the host.

Insights into the life cycles of *Haplosporidium* spp. can also be gained by examining the site and morphology of the early infection stages. Evidence is available only for oyster parasites, and it is not clear whether the initial site of infection is the epithelium of the gill or of the gut because infections have been found in only one or the other of these sites. Gut infections imply that the parasite is associated with food whereas gill infections imply that the parasite penetrates as a naked cell from the mantle cavity.

The hypotheses of (1) an intermediate host that ingests spores and (2) infections initiated by a naked cell or by a stage emerging from an oyster food organism are difficult to reconcile. Organisms that ingest spores are unlikely to be ingested by oysters, and organisms ingested by oysters are unlikely to be capable of ingesting spores. An intermediate host that ingests spores and releases a naked-cell stage into the water may be possible, but no such life cycle is known for the spore-producing protozoa. However, the discoveries made by Wolf and Markiw (1984) about the life cycle of *Myxosoma cerebralis* indicate that hypotheses should not be constrained by known life cycles.

Research on the life cycles of *Haplosporidium* spp. should be directed toward determining the fate of spores and identifying the water-borne infective stages. Histological sections of many estuarine organisms have been examined for stages of *H. nelsoni*, but no intermediate stages have been found. This survey type of approach has been hindered by the large number of potential intermediate hosts, by our poor understanding of the normal histology of most estuarine organisms, and possibly by our inability to recognize the parasite even if we encountered it. New techniques developed for the diagnosis of human and veterinary diseases have overcome the latter two problems. Two techniques, nucleic acid probes and immunoassays, hold great promise for the diagnosis of oyster diseases and for the discovery of an intermediate host. Nucleic acid probes are highly specific and have been developed for some human parasites (Wirth et al. 1986), but this technique has not been attempted for oyster parasites. Immunoassay has been successfully used to identify many human and veterinary parasites (Kurstak 1986). A marker is bound to an antibody molecule made against the parasite. The marker may be a fluorescent molecule, an enzyme, colloidal gold particles, or other molecules. When the antibody binds to the parasite, the attached marker can be visualized by several methods. Immunoassays that utilize enzyme markers are most widely used for disease diagnosis; however, the immunogold–silver staining method is gaining usage (Springall et al. 1984; De Mey et al. 1986).

Enzyme Immunoassay Technique

Enzyme immunoassay (EIA) is sensitive for detecting antigen or antibody, rapid, and conservative in the use of reagents. Variations of the technique are used for different objectives and may employ a variety of enzyme–chromogen combinations. In EIA, antibodies detect and distinguish closely related antigens; EIA uses enzymes that accelerate specific chemical reactions and that can be detected by adding appropriate enzyme substrates. Enzyme substrates are utilized that change color during the reaction and may also precipitate from solution. The principle of EIA is the conjugation of an enzyme and antibody; after the immunological reaction occurs, substrate is added and the amount or occurrence of color change is measured (Kurstak 1986).

In the direct EIA (Figure 1A), an enzyme is conjugated to the primary antibody, i.e., the antibody produced in response to a specific parasite. Because antibodies to oyster parasites are not commercially available, the enzyme conjugation for a direct EIA must be done by the investigator.

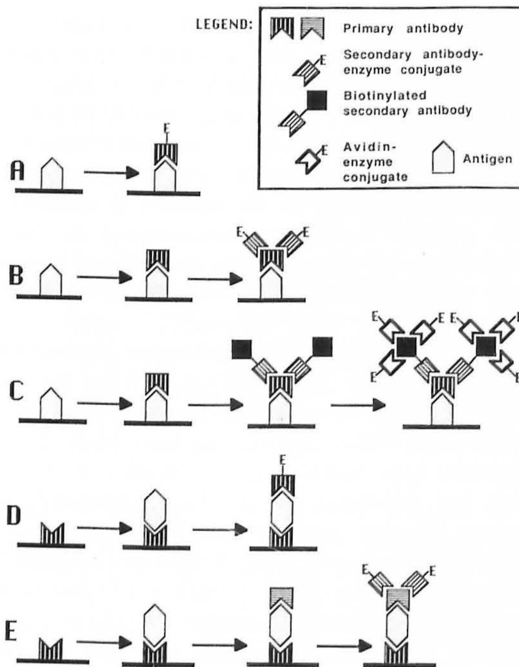

FIGURE 1.—Variations of enzyme immunoassays. **A.** Direct immunoassay. **B.** Indirect immunoassay. **C.** Indirect immunoassay with biotin–avidin bridge. **D.** Direct sandwich immunoassay. **E.** Indirect sandwich immunoassay.

Direct EIA could be used to detect solid-phase parasite antigen. For example, after primary antibody has bound to parasite antigen in a histological section, the addition of an enzyme substrate that precipitates as a colored substance would detect and locate the parasite.

The commercial availability of enzyme-conjugated goat antibodies produced against the immunoglobulin of various other species (e.g., rabbits) and the availability of kits containing required reagents has led to widespread use of indirect EIA. In this variation (Figure 1B), primary antibody is prepared by the investigator and is added to a histological section. If the specific antigen is present in the section, the primary antibody will bind to it. Next, an enzyme-conjugated secondary antibody, made against the immunoglobulin of the species in which the primary antibody was produced, is added, and it will bind to the primary antibody. As in the direct assay, the addition of a substrate that precipitates as a colored substance will detect and locate the parasite. The indirect EIA is more sensitive than the direct EIA because there are more binding sites for the secondary antibody. Although the sensitivity of the indirect method is about 10 times greater than the direct method (Kurstak 1986), the use of two antibodies in the indirect method increases the possibility of nonspecific binding and false positive readings. Sensitivity can be increased even further by combining a bridge technique with the indirect method. A common bridge is the avidin–biotin complex, which takes advantage of the affinity of avidin for biotin. Commercial kits are available that contain biotin-conjugated antibody and avidin-conjugated enzyme. The assay sequence is primary antibody, biotin-conjugated secondary antibody, and avidin-conjugated enzyme (Figure 1C). The affinity of avidin for biotin results in more bound enzyme and greater sensitivity than in the indirect method without the bridge. This increased sensitivity is illustrated diagrammatically by comparing the amount of enzyme in Figure 1A–C.

A variation called the sandwich EIA may be useful for screening many candidate intermediate hosts for the presence of parasites; however, a positive color change requires more than 10 ng/mL of antigen, an amount perhaps not obtainable from a small intermediate host. In this variation (Figure 1D), primary antibody is adsorbed onto a solid surface, usually the wells of a 96-well microtiter plate. Homogenized tissue from the assay organism is added to the wells and, if parasite antigen is present, the antibody will bind and remove it from solution. The wells are washed, and primary antibody–enzyme conjugate added. The antibody will bind to the parasite antigen present and, if a nonprecipitating substrate is added, the intensity of color in the supernatant will be proportional to the amount of parasite antigen. An indirect sandwich EIA, with a bridge method if desired, can be utilized to increase sensitivity (Figure 1E), but the two primary antibodies must be prepared in different host species or the enzyme-conjugated secondary antibody may bind to the adsorbed primary antibody and yield a false positive result.

Monoclonal versus Polyclonal Antibodies in EIA

Monoclonal and polyclonal antibodies each have advantages and disadvantages for application in EIA (Kurstak 1986; Goding 1987); the choice depends upon the objective of the assay and the availability of facilities and personnel. Antibody specificity is important when EIA is used in the search for an intermediate host. Antibodies recognize epitopes, single antigenic deter-

minants that combine with the antibody paratope. Polyclonal antibodies react with many different epitopes on an antigen; monoclonal antibodies react with a single epitope on an antigen. When the same epitope structure is located on different antigens, a monoclonal antibody cannot distinguish these antigens. Polyclonal antibodies, on the other hand, can distinguish such antigens because their specificity is the result of a unity of hundreds of different clones, and cross-reactions will be random and diluted (Goding 1987). Small changes in the structure of an antigen may have no effect on the binding of polyclonal antibodies because there will always be a subpopulation of paratopes that recognizes some epitopes on the antigen. In contrast, monoclonal antibodies may no longer be able to recognize the changed antigen (Handman and Mitchell 1986), a disadvantage in life cycle research because stage-specific antigens exist in the protozoan groups that have been studied in detail (Handman and Mitchell 1986). For example, monoclonal antibodies that recognize malaria sporozoites in mosquito vectors do not recognize merozoites of the same species in the mammalian host (Yoshida et al. 1980; Zavala et al. 1982), and monoclonal antibodies that recognize merozoites do not recognize sporozoites (Freeman et al. 1980). In contrast, monoclonal antibodies that recognize surface antigens of *Leishmania major* amastigotes and promastigotes reacted with antigens shared by both parasite stages (Alexander and Russell 1985). Monoclonal antibodies have been used effectively in the diagnosis of protozoan diseases (Handman and Mitchell 1986; Wirth et al. 1986); however, because of the possibility of stage-specific antigens, a polyclonal antibody may be better than a monoclonal antibody to detect a stage of an oyster parasite in an intermediate host. Wolf and Markiw (1984) used a polyclonal antibody to identify the intermediate host of the myxozoan *Myxosoma cerebralis*. A monoclonal antibody that is responsive to a single epitope on a parasite in oysters may not react with another stage of the parasite in an intermediate host; however, a mixture of monoclonal antibodies sensitized to various parasite epitopes may be effective. The production, testing, and identification of a monoclonal antibody with high affinity and low cross-reactivity is a long procedure; however, once obtained, the monoclonal antibody can be produced indefinitely, and the unreliability of antiserum production can be avoided.

For the preparation of polyclonal antibodies, a highly purified antigen is needed, and this requirement has hindered the development of immunoassays for oyster parasites. Contamination by host tissue will render the antibody useless for diagnosis because the antibody will bind to host tissue and yield false positives. Adsorption of the antibody with host tissue may remove the undesirable antibody, but it may also reduce the activity of the desirable antibody. Contamination with host tissue may be less important if the antibody is to be used to search for parasite antigen in another host species, but generally, the goal should be to obtain the purest antigen. Antigen purity is not as important in monoclonal antibody production because antibodies to host tissue can be discarded, but it is critical that host antigen contaminants are not so abundant that they become immunodominant (Handman and Mitchell 1986). Parasite antigens purified by affinity chromatography with monoclonal antibodies could be used to produce highly specific polyclonal antisera for use in life cycle research (Goding 1987).

Examples of Immunoassays

Research on haplosporidan life cycles at the Virginia Institute of Marine Science has focused on *H. costale* because the organism sporulates regularly every spring and large numbers of spores are present in each gaper (Andrews 1984). Spore-laden oyster tissue was fed to various oyster scavengers, and then the tissues of the scavengers were examined for parasite antigen by EIA. The sporoplasm is the cell that initiates an infection in the next host, so spores must be disrupted to expose the sporoplasm epitopes to the animal in which primary antibodies are to be produced.

Spores of *H. costale* were concentrated first by autodigestion of macerated oyster tissue in large glass beakers. Then, spores were washed three times in sterile seawater and disrupted by shaking at high speed with 0.1-mm glass beads. Spore wall material was not separated from sporoplasm material. Total protein was concentrated by dialysis to 0.145 mg/mL and mixed 1:1 with Freund's complete adjuvant. One milliliter of this suspension was injected intramuscularly into each of three rabbits. After 14 d, spores were disrupted again, and 1.0 mL of spore suspension without adjuvant was injected subcutaneously into each rabbit. The rabbits were bled by cardiac puncture 5 d later, and serum was separated from the clot by incubation for 1 h at room temperature and overnight refrigeration. Serum was adsorbed with homogenized *H. costale*-free oyster tissue, mixed 1:1, overnight at 5°C to remove any possible

oyster tissue antibodies. Antigen–antibody complexes were removed by centrifugation, and the serum was filtered, divided into aliquots, and frozen at −21°C. The assay used was an indirect biotin–avidin immunoassay with peroxidase as the enzyme. I used a Vector Laboratories commercial kit that contained biotin-conjugated goat antirabbit immunoglobulin G (IgG) and avidin–peroxidase conjugate. The substrate was hydrogen peroxide with a chromogen that turned blue–black after the reaction was completed; fast green was used as a counterstain. The antigen was *H. costale* in paraffin sections of oyster tissue preserved in 4.0% formaldehyde solution. The assay revealed *H. costale* spores in infected (Figure 2A) but not in uninfected oyster tissue (Figure 2B); reaction with *H. costale* plasmodia was weak. The antibody did not cross-react with spores of *H. nelsoni* or with spores of *Haplosporidium* spp. from the mud crab *Panopeus herbsti* and the naval shipworm. The failure of the antiobdy to recognize plasmodia indicates that epitopes of the spore wall and plasmodia are not similar; this result reinforces the need to isolate plasmodia or sporoplasms as well as spores for antibody production. Roubal and Lester's results (in press) in an immunofluorescent test for *Marteilia sydneyi* were similar: sporonts reacted but the sporoplasm did not. The disruption of *H. costale* spores and the purification of sporoplasm antigen have been hindered to date by an inability to obtain a sufficient number of spores.

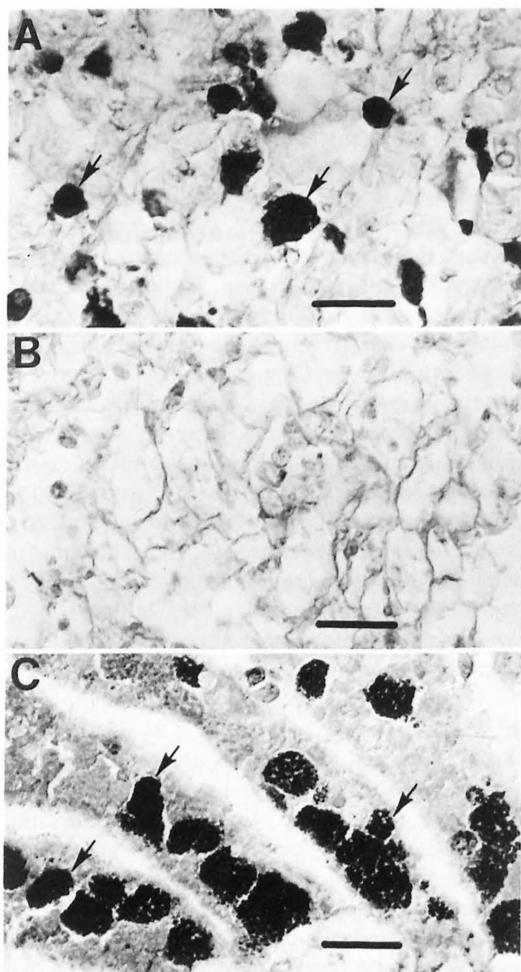

FIGURE 2.—Examples of immunoassay results. **A.** Tissue of oyster infected with *H. costale*. Arrows show positive reaction with sporocysts containing mature spores. Bar = 40 μm. **B.** Uninfected oyster tissue. Bar = 40 μm. **C.** Naval shipworm gills infected with *Haplosporidium* sp. Arrows show positive reaction with sporocysts. Bar = 80 μm.

For the characterization of *Haplosporidium* sp. of naval shipworms, an immunoassay was developed that used colloidal gold as the marker. Spore purification and primary antibody preparation were similar to that described for *H. costale* except that spores were disrupted in a sonicator and the Ribi adjuvant system was used instead of Freund's adjuvant. A Janssen Life Sciences Auroprobe-LM commercial kit was used that contained 5 nm colloidal gold bound to goat antirabbit IgG. Silver stain reagents were used to enhance the visibility of the colloidal gold. The results of one assay for spores in paraffin sections of naval shipworms are shown in Figure 2C. Strong positive reactions occurred with spores; the background staining with the colloidal gold assay was less than with the enzyme assay.

Immunoassays should increase the chance of delineating the life cycle of *H. nelsoni* or any of the parasites related to it. Assays should be developed for both monoclonal and polyclonal antibodies. Monoclonal antibodies obviate many of the problems of antigen purity that are associated with the production of polyclonal antibodies, and monoclonals or mixtures of monoclonals will be useful in many aspects of oyster disease research. Polyclonal antibody may be more useful in the search for intermediate hosts because of the potential for stage-specific antigens.

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