Evaluation of immunological techniques for host fish identification, and cryopreservation of embryos for conserving rare freshwater mussels

by

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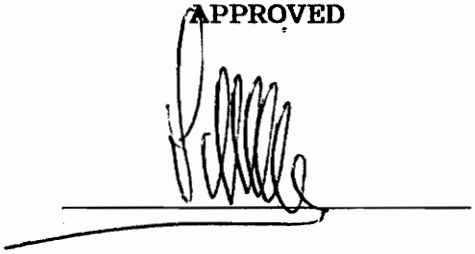

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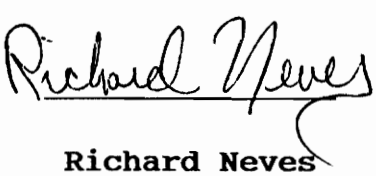

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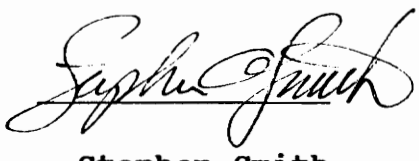

Stephen Smith

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Blacksburg, Virginia

Evaluation of immunological techniques for host fish identification and cryopreservation of embryos for conserving rare freshwater mussels (Mollusca:Unionidae)

by

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(ABSTRACT)

Glochidia (larvae) of freshwater mussels are obligate parasites which attach to and become encysted in the gills or fins of host fish species. The immune responses of the host fish to the parasite affects the susceptibility of the fish to glochidia of different mussels. The immune response provides an opportunity to identify which fish species are hosts. The number and variety of mussels in rivers and lakes has sharply declined since the last century due to various anthropogenic factors, and some mussels species are facing extinction. It is an urgent task to preserve these vanishing mussels, or extinction will be inevitable.

An attempt was made to develop an assay, using the immunological response to glochidia, to screen fish species for appropriate hosts. This would facilitate the production and rearing of juveniles. In order to design these assays, reagents such as anti-immunoglobulins which can react with antibodies from many different fish species have to be

developed. This work was carried out to develop such reagents. Host and non-host fish were immunized with killed bacteria (Brucella abortus) to study their humoral immune response to an antigen. All fish were able to respond well, as measured by agglutination and Western Blot assays. Antibodies produced by the Brucella injections were used to stimulate anti-fish immunoglobulins in goats, and the antisera were tested for their ability to recognize immunoglobulins from different host fish species. The specificities of these reactions were compared to the reactivity of Protein A. Goat antisera were able to cross-react with different fish antibodies, but it was found that Protein A was a more suitable reagent. Protein A is seemingly suitable to identify the host-fish species and could be used as a reagent for the serological diagnosis of various fish diseases.

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I dedicate this paper to my wife and son for encouraging me to pursue the degree.

Finally, I especially thank my parents in China for their support and tolerance of the long-time separation from us.

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Introduction

Glochidia of Freshwater Mussels

Glochidia (larvae) of freshwater mussels are obligate parasites on specific fish species (Zale and Neves, 1982). Most glochidia of mussel species attach to the host-fish after they are released into the water by gravid females. The site for attachment may be either the fish gills or fins (Meyers et al. 1977). Once glochidia attach to an appropriate host fish, they become encysted, obtain nutrition from the blood, metamorphose into the juvenile stage, and drop from the fish to start the free-living benthic stage of their life cycle (Isom and Hudson, 1984). Specific host fish species play a significant role in the reproduction of freshwater mussels. There are some important factors which may influence the successful attachment, survival, and transformation of glochidia. Release of glochidia from gravid females is induced by temperature, diurnal changes, and physical stimulation by fish (Cheng, 1988). However, the susceptibility to glochidiosis by fish species is unknown, and needs more research. It is believed that the most likely

factors affecting susceptibility to glochidiosis are the immunological responses of host fish (Reuling,1919; Arey,1932; Zale and Neves, 1982; Bauer, 1987). Previous research has demonstrated that fish reinfested with glochidia can exhibit strong resistance to the parasites, sloughing them off from the gills (Reuling, 1919; Arey, 1923, 1932). Further studies showed that similar immunological reactions occur in non-host fish species that slough off glochidia (Meyers and Millemann, 1977; Neves et al. 1985). Research conducted with fish parasites other then glochidia support the hypothesis that the most logical defense against them is the fish's immune system (Barriga, 1981; Manning and Mughal, 1985). Mechanisms that will increase the chances of glochidia contacting fish are physical adaptation (e.g. larval thread), physical appearance of conglutinates, and the location of mussel beds in areas where the fish host species occur (Kat, 1984). Based on the mussel species, glochidia may attach to the gills or fins of fishes (Meyers and Millemann, 1977). Attachment to the highly vascularized gill may lead to a faster immune rejection of the parasite, as opposed to attachment to the more peripheral areas of the fins (Young et al. 1987). After contact with a host fish, the glochidia must be able to survive the conditions of encystment. Many studies have demonstrated that survival of glochidia on an appropriate host fish is dependent on the immunological history of the host (Reuling, 1919; Arey,

1932; Barriga, 1981; Zale and Neves, 1982; Manning and Mughal, 1985). As mentioned previously, many appropriate host species exhibit an immunological resistance to reinfestation with glochidia (Reuling,1919; Arey, 1923,1932; Meyers and Milleman, 1977; Bauer and Vogel, 1987). The amount and duration of the immune response may vary with the number of glochidia involved in a second infestation and the species of fish (Arey, 1932). Not all studies agree with the immune rejection of glochidia. The results of studies by Young et al.(1987) suggested that there is little or no immune response to a second infestation. They successfully reinfected juvenile brown trout (Salmo trutta) with glochidia of Margaritifera margaritifera. However, it is possible that their contradictory results are due to differing physiological histories of the fish used in the tests.

The experiments of Manning and Mughal(1985) showed that young fishes are immunologically less mature and have overall weaker immune responses to parasites. Therefore, optimal glochidial parasitism on appropriate host species would be expected with younger and not previously exposed hosts. The immunological response of non-host fish, added to the fact that they are not the optimal host, leads to glochidial death if they attach to these fishes, particularly if previous exposure had occurred. Arey (1923) found that the immune response of non-host fishes to glochidia was due to non-

specific immunity, whereas the immune response of reinfested host fishes was due to acquired immunity. Under both situations, fish sloughed off the glochidia in different manners. Glochidia sloughed off by non-host fish is a faster response than the responses of host fish. Direct cytolysis of the sloughed off glochidia or of dead glochidia (Neves et al.1985) is often involved in the natural immunity phenomenon (Arey, 1923). In contrast, the acquired immune reactions to glochidia are weaker, slower and rarely cytolytic in nature (Arey, 1932; Meyers and Millemann, 1977; Young et al. 1987). In addition to both natural and acquired immunity, a variety of non-specific defense mechanisms such as those carried out by phagocytic cells can destroy attached glochidia (Meyers et al. 1980; Finco-Kent and Thune, 1987; Grayson et al, 1987; Siwicki and Studnicka, 1987). These studies suggest that glochidia attach to an appropriate host fish, become encysted in order to protect themselves from attack by the immune response, and obtain nutrients from the host fish to transform to juvenile mussels. Once ready, they excyst from the host fish (Kat, 1984; Neves et al. 1985). The ability of glochidia to withstand the host's immune response may depend upon the genetic background of mussel species since some glochidia on a particular host will die while other glochidia will survive (Arey, 1932). Isom and Hudson's (1984) experiment indicated that some "essential" component in fish blood is

necessary for initiation of glochidial transformation. This component seemingly occurs in the blood of all fish tested. In vitro studies by these investigators indicated that the use of growth medium without fish plasma did not lead to transformation. Similarly, transformation did not occur when fish plasma was replaced with other sera. Glochidia may attach and transform to juvenile mussels for a period of time ranging from days to weeks depending on water temperature (Kat, 1984).

Immunology of fish

In general, the essential immunological mechanisms of fish are somewhat similar to those in higher vertebrates, but clear differences exist between lower and higher vertebrates (Ellis, 1982). Comparing the humoral immune responses, five distinct immunoglobulin (Ig) isotypes exist in mammal antibodies, such as IgM, IgG, IgA, IgE and IgD including a variety of subisotypes. In contrast, only IgM-like molecules have been found in fish. This IgM-like fish antibody is a tetramer, whereas the mammal IgM is a pentamer. IgM and IgG have a different sedimentation coefficient and therefore have

often been referred to as 19s and 7s antibodies, respectively. Sequential immunization studies in fish demonstrated that 19s-like and 7s-like Ig are both produced by fish and they may possess some capacity for immunological memory (Cushing, 1970). Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) analysis of fish mucus IgM showed two bands of 70,000 and 25,000 daltons, representing the heavy and light chains respectively (Lobb and Clem, 1981, 1986, 1988; Vallejo, 1992). The total molecular weight of fish IgM in SDS-PAGE is approximately 95,000 daltons when associated covalently with disulfides.

Fish not only express humoral immune responses, but also express cell-mediated immune responses (CMI) and have an active complement system. The immunological response to glochidia may be the basis for host fish specific rejection (Isom and Hudson, 1984). Early studies on the immune responses to glochidia showed that a variety of immunological reactions such as humoral, cell-mediated, complement-mediated, and non-specific phagocytosis exist, but the most common immune responses to glochidia are humoral and cell-mediated immune reactions (Meyers et al. 1980; Bauer and Vogel, 1987; Wood and Matthews, 1987). Undoubtedly, fish have the capability to produce a variety of antibodies after stimulation with antigens of different parasites (Orr et al. 1969; Cushing, 1970; Barriga, 1981; Wood and Matthews, 1987).

Meyers et al. (1980) used two kinds of salmon, coho salmon (Oncorhynchus kisutch) and chinook salmon (O.tshawytscha), infected with glochidia of Margaritifera margaritifera, to study the antibody response. Their results suggested that both coho salmon and chinook salmon can generate serum antibodies against glochidia, as demonstrated in precipitation tests, although coho salmon expressed more resistance to glochidia than chinook salmon. The reliability of the precipitation test to quantify antibody levels has been questioned; therefore, it is difficult to assess whether both fish species produced similar amounts of specifically induced antibodies or whether the precipitating antibodies were mainly other natural antibodies in the sera of immunized fish. Similar experiments were conducted by Bauer and Vogel (1987) on brown trout infected with Margaritifera margaritifera, where antibodies against glochidia were detected in the serum of infested host fishes after exposure to glochidia.

Natural antibodies have been found in normal fish sera, and like the specific antibodies, have the same ability to agglutinate hemocytes and precipitate with antigens of bovine serum albumin (BSA) (Hodgins et al. 1967). Therefore, in addition to specific immune reactions affecting the glochidial parasite, the non-specific immune response represented by natural antibodies should be considered in the rejection of glochidia (Hodgins et al., 1967). Non-specific immune

responses like phagocytosis by neutrophils (Finco-Kent and Thune,1987), and macrophages (Arme and Walkey 1970; Bayne, 1990), as well inflammatory reactions (Barriga, 1981), may also be involved since they are efficiently used by host fishes to attack parasites.

The encystment of Lampsilis anodontoides on the gills of largemouth bass (Micropterus salmoides) has been observed by Arey(1932). At the site of encystment, the cyst was surrounded by eosinophils presumably in response to the parasite's presence. Eosinophilic cells have been shown capable of destroying parasite tissue (Barriga, 1981). A similar phenomenon has been described by Meyers et al. (1981), where eosinophils have been observed at the sites of glochidial cysts. Apparently, this form of eosinophil- based CMI response can be triggered by glochidia in a host fish.

In general, humoral and cell-mediated immunities are most likely the primary immune reactions against glochidia, although natural antibodies, natural killer cells (Evans and Cooper, 1990) and the complement system may play certain functions in immunity to specific antigens like glochidia.

Brucella abortus

Brucella abortus is a gram-negative coccobacilli and is a facultative intracellular parasite. Because it resides inside macrophages, this organism can escape the attack of the humoral response. The cell wall of Brucella contains lipopolysaccharide (LPS) which has three main components: the O-side chain, the core polysaccharides and the lipid A. If the LPS of Brucella contains the O-side chain, the strain is usually smooth and virulent; if the O-side chain is not present, the strain is usually rough and avirulent.

Three strains of Brucella abortus, strain 2308, strain 19, and strain RB51, have different O-side chain lengths. Among these strains, strain 2308 is highly virulent, strain 19 has been widely used as a vaccine strain in the United States, and strain RB51 has no O-side chain (Buhrman, 1989, Sriranganathan et al., 1991). In SDS-PAGE gel analysis, the position of the O-side chain is found in the upper molecular weight and usually appears as a "smear" on the developed gels. Considering its antigenicity, the O-side chain attached to the LPS is highly immunogenic and therefore, can be used as an antigen to elicit immune reactions. Usually, the tube agglutination test with whole Brucella organisms is used to measure the immune reaction to Brucella O-chain. Western Blot

analysis is also useful to detect the O-side chain of the Brucella LPS.

Finstad and Good (1964) immunized sea lamprey (Petromyzon marinus) with killed Brucella abortus cells ($1x10^9$ cells or $1x10^7$ cells in 0.5ml per animal) to evaluate the evolution of the immune response. Comparing several antigens, agglutinating antibodies were detected 14 days post-immunization in immunized lampreys. After thirty-two days from secondary stimulation, the titer of the agglutinating antibodies had increased and was higher than those stimulated by other antigens such as purified hemocyanin, bovine gamma globulin, and bacteriophage T2 (Escherichia coli). They demonstrated that Brucella abortus, following both primary and secondary stimulation, can evoke strong specific immune responses in lampreys. Similar tests were done by Pollara et al. (1970) in lampreys (Petromyzon marinus) to evaluate the evolution of the immune response. The animals were immunized with human "O" red cells and killed Brucella cells. The lamprey produced antibodies against both antigens, reaching agglutinating titers of 1:1280 with both antigens. Ingram and Alexander (1980) infected brown trout (Salmo trutta) with Salmonella typhimuri LPS, with and without adjuvant, to compare the immune responses to different antigens in brown trout. High titers of agglutinating antibodies and complement-fixing antibodies, but no precipitating antibodies

were produced with adjuvant. These experiments indicated that Brucella abortus cells, especially its LPS, can act as a good antigen for fish to elicit a high fish humoral immune response.

Protein A

Protein A is a natural anti-antibody that can be isolated from the cell wall of most strains of Staphylococcus aureus. Staphylococcal Protein A can specifically interact with immunoglobulins. Some of the unique properties of Protein A and their applications were summarized by Surolia et al. (1982). The unique biological property of Protein A is characterized by its ability to interact with the Ig's of many species including IgG and IgM . Protein A does not bind avian IgG and gives only a weak reaction with ruminant IgG. The ability of goat and sheep IgG antibodies to bind Protein A can be markedly enhanced by immune binding to immobilized antigen or hapten.

^{125}I-labelled Protein A is also useful for the detection of antibody (and indirectly of antigen) on the cell surface. Gold-labelled Protein A can be used in electron microscopy to

label antigen-antibody sites on lymphocytes, platelets and other cells. Recently, Protein A labelled with FITC molecules and gold particles has been used in both light and electron microscope immunocytochemistry.

There are only two studies dealing with Protein A in lower vertebrates. Hastings and Ellis (1988) used Protein A to detect the antibodies in rabbits following immunization with native extracellular products (ECP) of Aeromona salmonicida. Therefore, Protein A was being used to detect mammalian immune response in this study. In the other study, Cook et al. (1991) applied Protein A gold as a marker to identify gonadotropin-releasing hormone receptors on gonadotrophs and somatotrophs of the goldfish in an electron microscope study. Therefore, it appears that Protein A has not been used to detect the humoral immune response in fish.

General project goals

Due to various pollutants such as arsenic, cadmium, chlorine, copper, iron, mercury and zinc; channelization and dams; commercial harvest; wood product wastes; organic enrichment; acid mine waste; pesticides and radionuclides; habitat destruction; and competition with introduced exotic species, a dramatic decline of freshwater mussels has occurred

in the last century (Fuller,1974). In addition to affecting the mussels directly, these factors also cause the loss of host fishes for freshwater mussels. Some measures such as development of an artificial medium to culture glochidia in vitro (Isom and Hudson, 1982; Keller and Zam, 1990) have been undertaken to save declining mussel species. However, there are problems with this method; for example, not all freshwater mussels can be successfully transformed by this method, and if the glochidia are cultured by the artificial medium in vitro, the genetic integrity of the species may be threatened. Although specific serum components are used by the glochidia to metamorphose and excyst from host fish, the nature of these chemical components is still not clear. It is likely that a component of fish plasma is essential since glochidia will fail to transform to juveniles if fish plasma is not present in the culture medium.

There have been attempts to identify host fish species for the various freshwater mussel species, in order to successfully propagate juveniles for mussel conservation. Based on knowledge of the immunological reactions of host fish to glochidia, it may be possible to use serological methods to detect specific reactions and therefore detect host fish species. One practical problem, however, is that many serological methods such as agglutination and precipitation are not very sensitive, and there are hundreds of fish species

to be tested. Due to species differences, immunoglobulins from one fish species cannot be detected with anti-immunoglobulins directed against other fish species. It becomes essentially impossible to prepare antiserum for every host and non-host fish species to detect antibodies to each freshwater mussel species. However, if Protein A reacts with the Ig of all fish species, a test can be developed to screen sera from any fish species for specific activities. Both host and non-host fish species can produce antibodies to the glochidia of freshwater mussels; the only difference is the degree of immunity (Meyers et al. 1980). The immunity in fish completely depends on the temperature and physical conditions; if the temperature is controlled, it is possible to make the non-host fish species become host fish species. The goal is to develop one serological method that would identify all host species so that these fishes can be used to increase the populations of endangered and threatened mussel species.

Cryopreservation for freshwater mussels

Investigators have identified host-fish species for glochidia of some mussel species, but most mussel species have not been studied. Although these studies are biologically sound, they require considerable time to be completed. Renard (1991) used methanol and sucrose as cryoprotectants to study the effects of cooling and freezing tolerances on embryos of the Pacific oyster, Crassostrea gigas. Based on this experiment and knowledge of cryobiology, certain solutions and cryoprotectants are available that could lead to protection of living embryos of mussels from injury due to freezing during cryopreservation. It may be possible to successfully store the embryos of mussels in liquid nitrogen at -197° C. If cryopreservation is feasible the genetic materials contained within these rare populations of mussels can be preserved. Also, glochidia could be available year-round for in vitro culture, and glochidia would be available at all times for host-fish infestation studies in the laboratory. This would greatly contribute to the preservation of mussel species.

Objectives

The main objectives of this study are:

1) to determine the immunological reaction of host fish and non-host fish species to killed Brucella strain 19.

2) to determine whether anti-immunoglobulin against one fish species can cross-react with specific antibodies produced by other host fish and non-host fish species. These reagents would be used to establish a serological model to screen fish species for their ability to host or not host glochidia of freshwater mussels.

3) to determine if Protein A can be used to detect fish Igs to eventually establish a reliable, simple and more sensitive immunological method to detect antibodies and specifically glochidia .

4) to develop a cryopreservation method to store the embryos of mussels at supercooling temperatures.

Materials and Methods

1. Animals

Rock bass (Ambloplites rupestris), from 30 to 150g in weight, served as the host fish species for glochidia of the rainbow mussel Villosa iris. This mussel species is endemic to the upper Tennessee River basin and has had host fishes determined in a previous study (Zale and Neves, 1982). These fish were collected with electro-shocking gear from Tom's Creek which has no mussels at McCoy, Virginia. Goldfish (Carassius auratus), from 700 to 800g, and common carp (Cyprinus carpio), from 70 to 500g, which are non-host fish for glochidia of mussels, were obtained from the campus pond at Virginia Tech by hook and line sampling. All fish were held in Living Streams at 18° C under identical conditions in the Aquatic Medicine Laboratory of the VMR-College of Veterinary Medicine. The Living Streams with rock bass, goldfish and carp were separately supplied with dechlorinated tap water, and water was changed as necessary. The goldfish and carp were fed a commercial pelleted feed once daily, while rock bass were fed live young goldfish. Fishes were maintained in Living Streams for 15 days prior to experiments.

2. Brucella abortus antigen

Brucella abortus strain 19 was used as the antigenic source for all experiments. Trypcase soy agar (TSA) plates were inoculated with B. abortus strain 19 and incubated in a 37° C incubator with 5% C02 atmosphere for 48 h. The bacteria were harvested with 8 ml of 0.85% sterile physiological saline, and the suspensions were washed with saline three times at 2000 x g for 10 minutes. The pellets were suspended in 10 ml saline and, in order to kill the Brucella, an equal amount of acetone was added and incubated with stirring at 37° C for 4 h. After incubation, the acetone-treated suspensions were washed with saline four times at 2000 x g for 10 minutes. Finally, the pellets were suspended again with 10 ml of saline and stored at 4° C. In order to ensure that all organisms were killed, a TSA plate was streaked with one drop of each of the suspensions, incubated at 37° C for at least 48 h, and observed for growth. Before treatment with acetone, it was determined that suspensions had 1.3×10^5 colony forming units (CFU) per ml. No growth was observed after acetone treatment.

3. Preparation of glochidia antigen of freshwater mussels

Glochidia of Villosa iris, which were collected from

Copper Creek,Scott County, Virginia, were homogenized with a homogenizer (Kinematica CH-6010 Kriens-Lu, Brinkman Instruments,Co.) on ice, frozen at -20° C, thawed and diluted 1:10 with physiological saline. This suspension was stored for later immunization of fish. Before use, the suspension was clarified by centrifugation at 2000 x g for 10 min, and the supernatant was used for the experiments.

4. Fish immunization

All fishes were bled before immunization (negative control), and sera were stored at 4° C. Individual fish were injected intraperitoneally (IP) with 0.5 ml containing 3.2 x 10^5 killed B.abortus strain 19 organisms mixed 1:1 with Freund's complete adjuvant (FCA) on day one. A second IP injection of 0.5 ml antigen mixed 1:1 with Freud's incomplete adjuvant (FIA) was given 14 days later. Before the injection, blood was taken from the caudal peduncles of fishes. Seven days later (day 21), 0.5 ml antigen was inoculated IP into all fishes without adjuvant. The fishes were bled before injection, and again 10 days later (day 31). Sera were isolated and aliquoted into 0.5 ml portions and stored at -20° C for subsequent experiments. Using a similar procedure, six host rock bass were immunized with the homogenized

glochidia of the mussel species.

5. Standard tube agglutination test to measure the titer of fish sera and the humoral immune response to B.abortus

The following procedure was followed:

a. A working solution of standard tube antigen was prepared by mixing one ml of B. abortus stock antigen (killed B. abortus St 19, SR 3-9101 from Center National Animal Disease Center of United States) into 99 ml phenol saline.

b. Test each serum against the working antigen solution starting with a 1:25 dilution of the serum by adding 80 ul of sera to 1.92 ml of antigen.

c. Make serum double dilutions ending with a dilution of 1:12,800.

d. Cover tubes and place into incubator at 37° C overnight.

e. Read test.

6. Isolation of specific anti-Brucella fish Ig.

In order to isolate Brucella-specific fish antibodies, killed B.abortus cells were reacted with the sera. This left

a bacterial suspension coated with specific fish Ig's.

a. Agglutination positive sera of three fish species were diluted 1:25 and 1:50, and mixed with the standard tube antigen as described before.
b. Agglutinated organisms were washed with sterile physiological saline four times at 2000 x g for 10 minutes.
c. Forty ul of 10mM Tris buffer pH 8.9 were added to the washed 1:25 agglutination pellet for SDS-PAGE. One ml of sterile saline was added to the 1:50 pellet, and store at 4° C. This dilution was used for goat immunizations.

7. Elution of the fish antibodies from the tube agglutination antigen.

Agglutination tubes with fish immune sera (rock bass, goldfish or carp) diluted 1:100, and with the standard St 19 tube antigen, were incubated at 37° C overnight. The pellets were washed at 2,000 x g four times for 10 min with 0.85% saline. The pellet was then treated with a solution of glycine -HCL buffer (23.57 g glycine, 8.2 ml concentrated HC, DW 950 ml) pH 2.8 for 2 min, centrifuged at 2,000 x g for 10 min, and the supernatant placed in a dialysis membrane tube

(M.W. cut off: 12,000-14,000; Fisher Scientific) and dialyzed against PBS, pH 7.2 with at least 6 changes for 24 h. In order to verify that the antibodies had actually eluted from the bacteria, the tube agglutination test was repeated with the eluted antibodies of rock bass, goldfish, and carp. If agglutinations with the eluted antibodies were confirmed, the eluted antibodies were stored at -70 ° C for 24 h, and then lyophilized.

8. SDS-PAGE and Western Blot for fish Ig.

a. 10% SDS-PADE was prepared according to " Gel electrophoresis of proteins" (Hames and Rickwood, 1981).

b. The samples (see [6]) were mixed with an equal amount of the 2x sample buffer (0.0625 M Tris buffer pH 6.8, 2.0% SDS, 5% 2-Mercaptoethanol, 10% glycerol, 0.001% bromophenol blue), and the samples boiled for 5 min.

c. Wells were loaded with 15 ul of the boiled samples.

d. The gel was electrophoresed with 50mA, 150V (Bio Rad Model 3000Xi) supplied with cool-cycling water (Haake A82) at 10° C for 1 h and 15 minutes.

e. The electrophoresed proteins were transferred onto nitrocellulose membranes (Micron Separations Inc.) at

125 V (Bio Rad Model 250/2.5) at 10° C for 2 h.

f. The membrane was placed into a blocking solution consisting of 2% bovine serum albumin (BSA) in Tris saline (TBS) pH 7.4 for 30 minutes after which the membrane was washed 5x in TBST (TBS + 0.05% Tween 20), and then washed 3x in TBS.

g. Rabbit anti-fish (Teleostei) whole serum (Sigma Chemical Co.) was diluted in TBS to 1:500, and the membrane was placed in this solution and left agitating overnight at room temperature.

h. The membrane was washed 3X in TBST, with agitation for 10 min at each wash.

i. The membrane was placed in a 1:500 TBS dilution of goat anti-rabbit IgG (whole molecule) conjugated with horseradish peroxidase (Organon Teknika Corp.) and incubated with agitation for 1 h at room temperature.

j. The membrane was washed 3 X TBST for 10 min each.

k. The membrane was developed in developing solution (60 mg 4-chloro-1-naphthol, 10 ml methanol, 100 ml TBS, 0.6 ml H202) for 10 min, and the reaction stopped by placing the membrane in distilled water.

9. Coomassie Blue staining of SDS-PAGE.

After the sample was electrophoresed, the gel was stained with Coomassie Blue stain solution (3.0 g Brilliant Blue, 100ml glacial acetic acid, 450 ml 100% ethyl alcohol, 450 ml distilled water) with agitating overnight at room temperature. The next day the gel was washed with destain solution (650 ml distilled water, 250 ml 100% ethyl alcohol, 100 ml glacial acetic acid) until all background color had disappeared.

10. Goat immunization with fish antibodies against St 19

Three goats, 75 to 85 lb in weight, were used for immunization. All goats were bled prior to each immunization. The 1:50 agglutination tube of fish antibodies against St 19, which had been stored at 4° C, was used for all immunizations of goats. One goat was inoculated with rock bass antibody, another with goldfish antibody, and another with carp antibody. The first injection for each goat consisted of 3 ml of the stored antigen mixed 1:1 with Freund's complete adjuvant at day 1 subcutaneously (SC); the second injection consisted of 3 ml with Freund's incomplete adjuvant in the same proportion at day 14 SC; the third consisted of 3 ml without adjuvant at day 21; and the fourth injection consisted of 3 ml without adjuvant at day 31. Blood was collected from each goat 10 days after the last injection. The sera were

separated, aliquoted in small amounts and stored at -20° C for further use.

11. Goat anti-strain 19 titers

Each collection of sera from goats was examined for specific anti-B.abortus antibody using the standard tube agglutination.

12. Goat anti-fish immunoglobulin antibodies

Antibodies to fish immunoglobulin in goat sera were detected by immunoelectrophoresis (IEP). Seven ml of melted 1% solution of agarose in Trizma Barbital Buffer pH 8.9 (Sigma Diagnostics) were pipetted onto a 75 x 50 mm microscope slide (Fisher Scientific Co.) to 1 mm thickness. The agarose was allowed to cool and gel at room temperature for 15 to 20 min and then placed at 4° C for an additional 15 to 20 min. Four holes of 3mm diameter were punched into the gel, and filled with 15 ul of the fish pre- and post-immunization serum. The gels were electrophoresed in Trizma-Barbital Buffer, pH 8.9, at 10 mA per gel for 45 min. After electrophoresis, three long troughs 45mm x 1mm were cut between the holes. The

troughs were filled with 0.75 ml goat antisera; the gel was placed in a humidified chamber, and left overnight at room temperature. If the gel did not show any reaction, it was further incubated submerged in 10% saline for 24 h.

13. Western Blot of goat antisera versus fish antibodies

The Western Blot method described under [7] was used, except that wells were loaded with sera of rock bass, goldfish and carp. The primary antibody consisted of each individual goat serum diluted 1:100 with TBS pH 7.2. After the anti-strain 19 fish antibodies were transferred electrophoretically onto the nitrocellulose membrane, the membrane was reacted with the primary diluted goat antisera with agitation overnight at room temperature. The secondary antibody was rabbit anti-goat peroxidase conjugated IgG (Organon Teknika Corp.) diluted 1:500.

14. Protein A recognition of fish antibodies (rock bass, goldfish and carp).

Protein A-Peroxidase conjugate (Sigma Chemical Co.) was dissolved in 1 ml 10 mM Tris, pH 8.0, and a working solution

of 0.1 ml dissolved Protein A solution in 20 ml 10 mM Tris was made. The wells in the 10% SDS-PAGE were loaded with the eluted antibodies of rock bass, goldfish, carp, and a positive control of affinity chromatography purified monoclonal mouse antibodies. The SDS was transferred onto nitrocellulose, and the membrane was incubated with the Protein A working solution for 2 h, washed 3 times with TBS, pH 7.2, and developed with the 4-chloro-1-naphthol developing solution.

The Protein A Western Blots were compared with similar Westerns in which either goat immune serum, or rabbit anti-fish serum were used.

Three comparative tests of Western Blot were used to determine whether Protein A, goat immune serum and rabbit anti-fish serum could detect the specific anti-Brucella reaction of the immune rock bass, goldfish and carp serum. For this purpose, wells of 10% SDS-PAGE were loaded with strain 2308 LPS, electrophoresed, and the antigens transferred onto nitrocellulose. The membranes were then treated individually with 1:100 dilutions of either rock bass, goldfish, or carp immune serum overnight with agitation at room temperature. The treated membranes with the individual fish immune serum were then reacted with either Protein A, goat antisera, or rabbit anti-fish serum. Several different fish sera were examined including affinity purified fish Ig, positive anti-A.salmonicida bass serum, whole Ig hybrid

striped bass serum Ig, and negative serum.

15. Western Blot to detect reactions between A.salmonicida positive antiserum and Protein A.

The Western Blot used was the same as described before [7], except that the antigens of A.salmonicida were loaded into 10% SDS-PAGE gel, and transferred onto the nitrocellulose membrane.

16. Using Protein A, goat antiserum against fish antibodies and rabbit anti-fish whole serum to detect positive antibodies against glochidia in Western Blots.

A 1:10 glochidia suspension mixed with 2x sample buffer was loaded on 10% SDS-PADE gel, and transferred onto nitrocellulose membranes. The membranes were then reacted with the positive fish antiserum against glochidia, and then reacted with 1) protein A-HRPO, 2) primary goat anti-fish antibodies such as goat anti-rock bass, goat anti-goldfish, and goat anti-carp, and with the secondary HRPO labeled rabbit anti-goat antibody, or 3) primary rabbit anti-fish whole serum and reacted with the secondary HRPO labeled goat anti-rabbit

serum.

17. Cryopreservation for glochidia of freshwater mussels

1. The early stage glochidia of freshwater mussels (Villosa iris) were exposed to 100% isopropanol, anhydrous (Sigma Diagnostics) for 30 sec and then to n-hexane (Sigma Chemical Co.) for 30 sec.
2. The treated glochidia were equilibrated in BD.20 vitrification solution containing 8.5 M ethylene glycol and 6% (w/v) bovine serum albumin (Steponkus, 1990) at room temperature for 20 min.
3. The equilibrated glochidia were placed in a cryophilization vial and rapidly plunged into liquid nitrogen at -197°C and kept frozen for 24, 48,and 72 h.
4. The vial was removed from the liquid nitrogen, and the embryos were equilibrated in BD.20 solution (5mM $CaCl_2$, 9mM $MgCl_2$, 10 mM $MgSO_4$, 3mM NaH2PO4, 68mM glutamic acid, 67mM glycine, 4mM malic acid, 0.2 mM sodium acetate; pH6.8) at 22 °C for 30 min.
5. Thawed glochidia were cultured in an 199 media (Sigma Chemical Co.) supplemented with 10% fish plasma, and viability of glochidia determined by movement of the glochidia under the microscope after the glochidia were cultured at 22°C for 24 h.

Results

I. Standard tube agglutination of rock bass, goldfish and carp sera

Both host-fish (rock bass) and non-host fish (goldfish and carp) exhibited an immune response with memory of similar strength to B.abortus strain 19-killed organisms. No agglutinations occurred with any of the pre-immunization (negative) sera. The agglutination titer after the first immunization (day 14) was 1:25 for both goldfish and carp, and 1:50 for rock bass (Table 1). The serum titer increased after the second immunization, and sharply increased after the third immunization, reaching very high titers of 1:6400 for rock bass, and 1:3200 for both non-host fish. The tube agglutination test indicated that all three fish species had a strong immune response to strain 19.

Table 1. Standard tube agglutination titers of fish sera after several immunizations with killed B. abortus cells.

Fish species	Maximum titer after immunization		
	1st(day 14)	2nd(day 21)	3rd(day 31)
Rock bass(host)	1:50	1:800	1:6400
Goldfish(non-host)	1:25	1:800	1:3200
Carp(non-host)	1:25	1:800	1:3200

II. Western Blot and Coomassie Blue stain for the second and third fish immune serum agglutinated with the standard tube agglutination St 19 antigen at 1:25.

Because Western Blots for the first post-immunization sera were not clear, the second post-immunization sera (day 21) and the third post-immunization sera (day 31) were agglutinated with standard tube agglutination St 19 antigen at 1:25 dilution for Western Blot (Figs. 1 and 2) and Coomassie Blue stain analysis (Fig. 3). The results showed that antibodies to anti-St 19 were contained in all immune serum. The bands which represented the antibodies against LPS antigen of B. abortus strain 19 appeared clearly, with the positive fish antisera in lanes 2, 4 and 6 (Figs. 1 and 2).

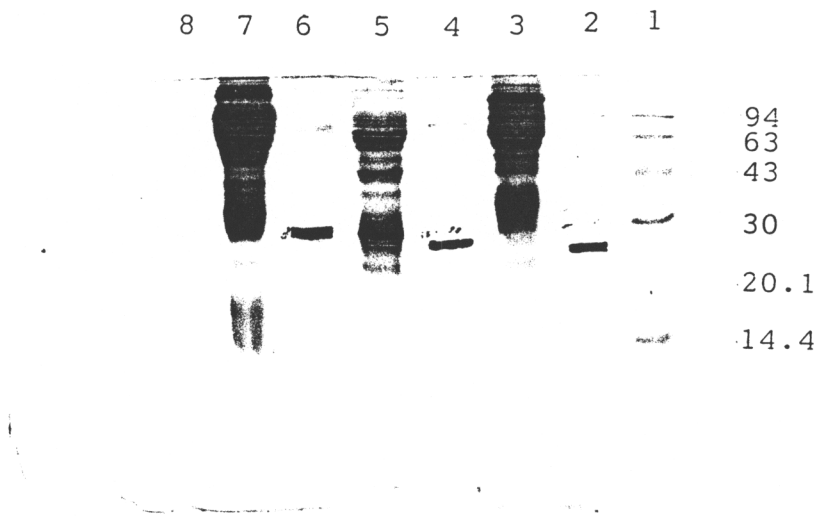

Fig. 1: Western Blot for the second fish immune serum agglutinated with standard tube agglutination St 19 at 1:25 dilution.

The primary antibody was rabbit anti-fish whole serum, and the secondary antibody was goat anti-rabbit serum-HRPO.

Lane 1, molecular weight (standards kd); lane 2, 4, and 6, the second fish post-immunization serum agglutinated with standard tube agglutination St 19 antigen at 1:25; lane 3, 5 and 7 pre-immunization serum of three fish species; and lane 8, St 19 acetone-killed organisms.

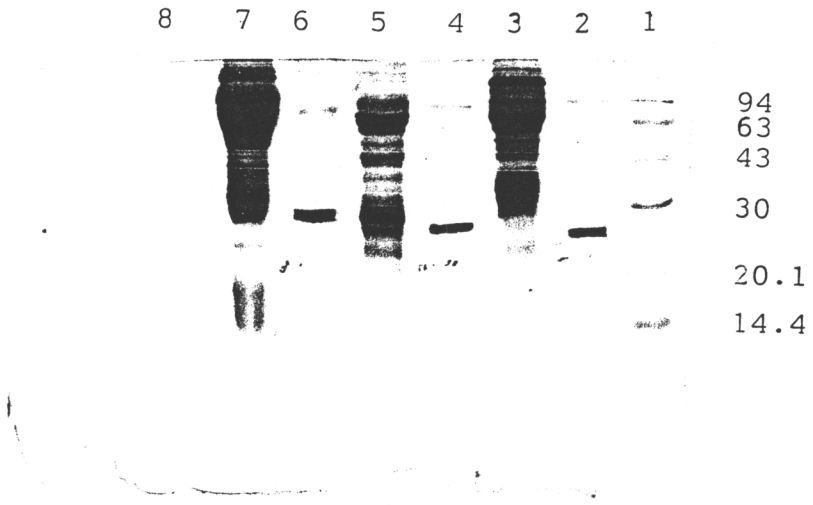

Fig. 2: Western Blot for the third fish immune serum agglutinated with standard tube agglutination St 19 antigen at 1:25. The primary antibody was rabbit anti-fish whole serum, and the secondary antibody was goat anti-rabbit serum-HRPO. Lane 1, molecular weight(kd); lane 2, 4, and 6, the third fish post-immunization serum agglutinated with standard tube agglutination St 19 antigen at 1:25; lane 3, 5 and 7 fish pre-immunization serum; and lane 8, St 19 acetone-killed organisms.

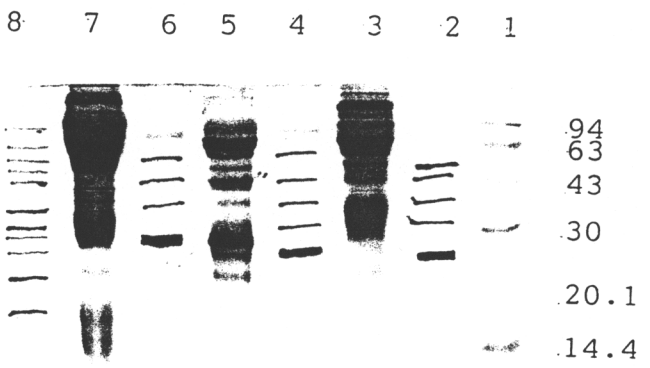

Fig.3: Coomassie Blue stain for the third fish post-immunization serum.

Lane 1, molecular weight (standard kd); lane 2, 4 and 6, fish post-immunization serum agglutinated with standard tube agglutination on strain 19 antigen; lane 3, 5, and 7, fish pre-immunization sera; and lane 8, St 19.

III. Tube agglutination of serum from goat serum immunized with fish antibodies and St 19 complex

Like the fish humoral response to killed St 19 antigen, the goats expressed a similar immune response to this organism. The titer of the goat sera was very low after the first immunization, only 1:50 in all goat sera (Table 2). However, after the second injection, the titers were higher, up to 1:400 (Table 2). The titer remained constant at 1:3200 after the third and fourth injections (Table 2). The pre-immunization goat serum had no detectable agglutination with the St 19 standard antigen.

Table 2: Standard tube agglutination of goat sera 14, 21, 28, and 36 days after initiation of immunization schedule.

Goat	Titer after immunizations			
	1st(day 14)	2nd(day 21)	3rd(day 31)	4th(day 36)
goat anti-rock bass	1:50	1:400	1:3200	1:3200
goat anti-goldfish	1:50	1:400	1:3200	1:3200
goat anti-carp	1:50	1:400	1:3200	1:3200

IV. The use of goat antisera to detect fish (rock bass, goldfish, and carp) antibodies against St 19 in Western Blots

Fish immune sera from rock bass, goldfish and carp were separated by SDS-PAGE, transferred onto the nitrocellulose membrane, and goat antisera used as the primary antibody. All goat antiserum; goat anti-rock bass, goat anti-goldfish, and goat anti-carp, expressed cross-reactivity with the three fish species. For example, serum from the goat which was immunized with rock bass immune serum could recognize goldfish and carp antibodies (Fig. 4). A similar phenomenon was observed with serum from the other two goats. No reactions were observed with goat pre-immunization sera.

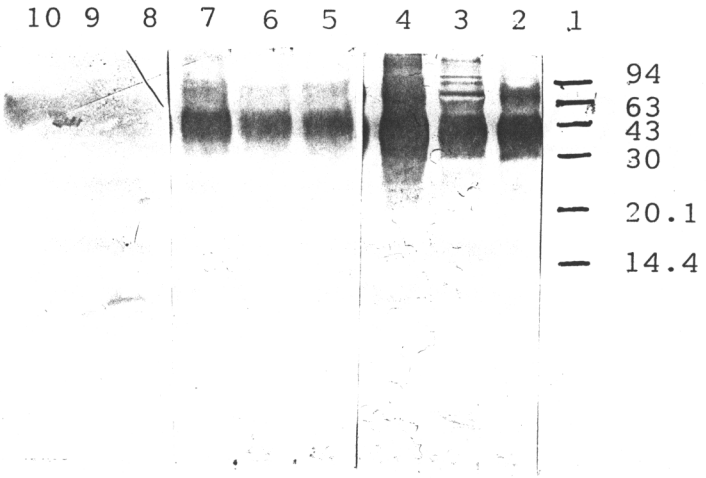

Fig 4: Western Blot of goat immune serum detecting fish antibodies.

Lane 1, molecular weight standards(kd); lane 2, 5, and 8, rock bass immune serum; lane 3, 6, and 9, goldfish immune serum; lane 4, 7, and 10, carp immune serum; goat anti-rock bass for lane 2, 3, and 4, goat anti-goldfish for lane 5, 6, and 7, and goat anti-carp for lane 8, 9, and 10.

V. Immunoelectrophoresis (IEP)

The characteristics of the precipitation bands, as seen in Fig. 5, 6, 7, and Table 3 (summarization of the precipitation reaction) indicated that anti-goldfish and anti-carp sera reacted with the serum of all 3 fish species. Reactions were usually strongest when a particular antigen was reacting with its corresponding antisera. In general, two major precipitation bands, one short and another long one, were detected.

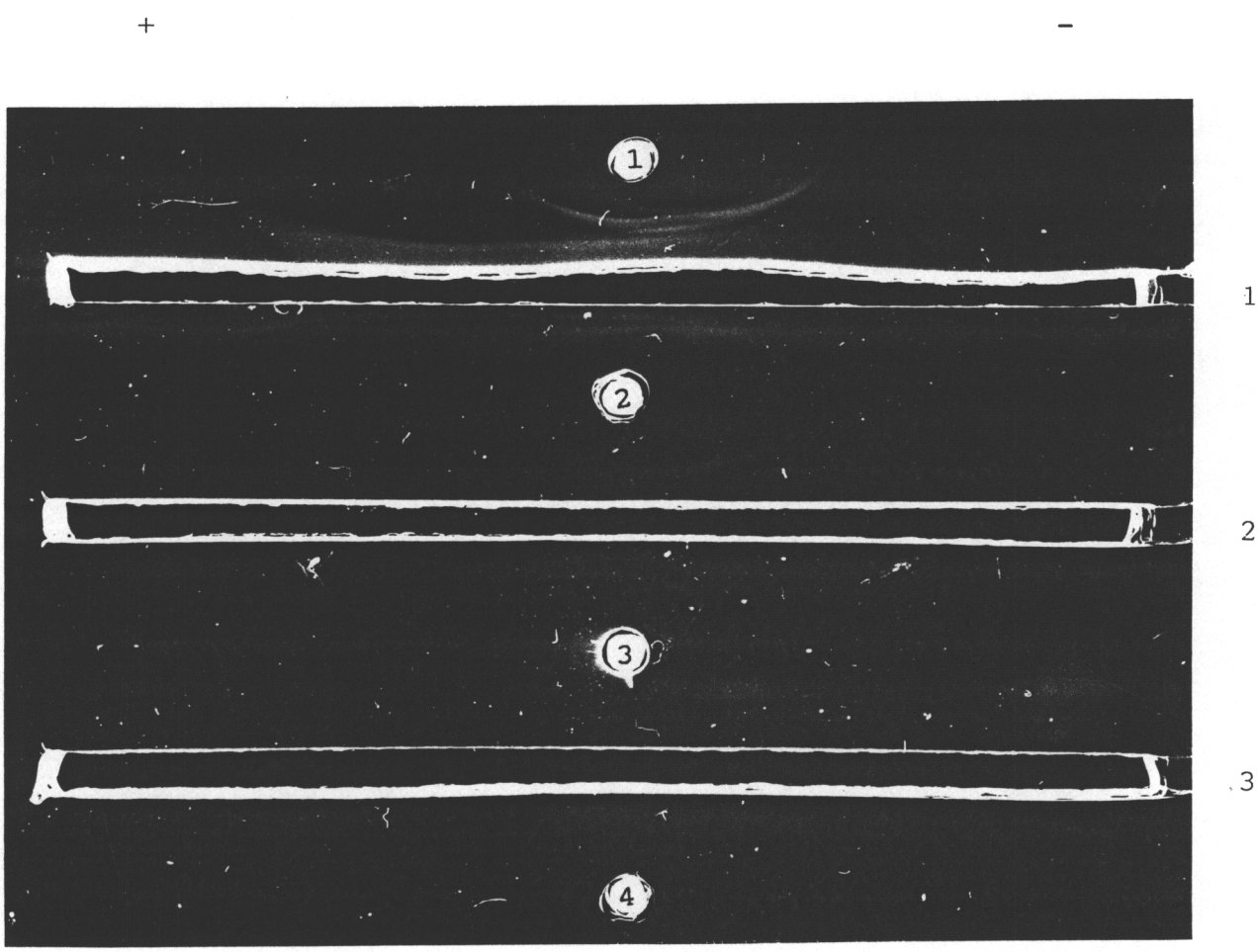

Fig. 5: Precipitation bands formed between goat antisera and antigen (fish pre and post-immunization serum).
Well 1, pre-immunization goldfish serum; well 2, post-immunization goldfish serum; well 3, post-immunization carp serum; and well 4, post-immunization rock bass serum. Trough 1, goat antiserum against goldfish immune serum; trough 2, goat to carp serum; and trough 3, goat antiserum to rock bass serum.

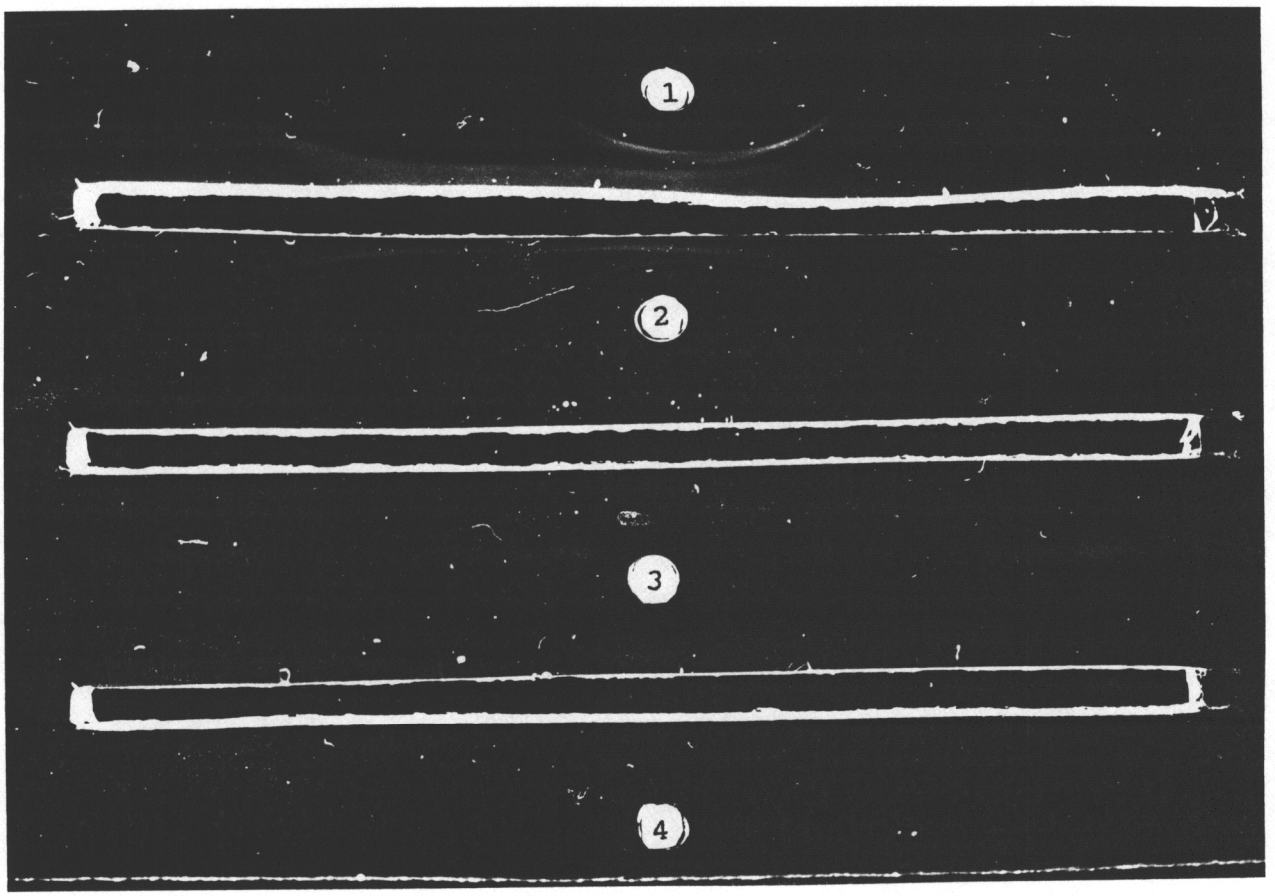

Fig. 6: Precipitation bands formed between goat antisera and antigen (fish pre- and post-immunization serum). Well 1, pre-immunization carp serum; well 2, 3, and 4, post-immunization carp, rock bass and goldfish serum, respectively. Trough 1, goat anti-carp antiserum; trough 2, goat anti-rock bass antiserum; and trough 3, goat anti-goldfish serum.

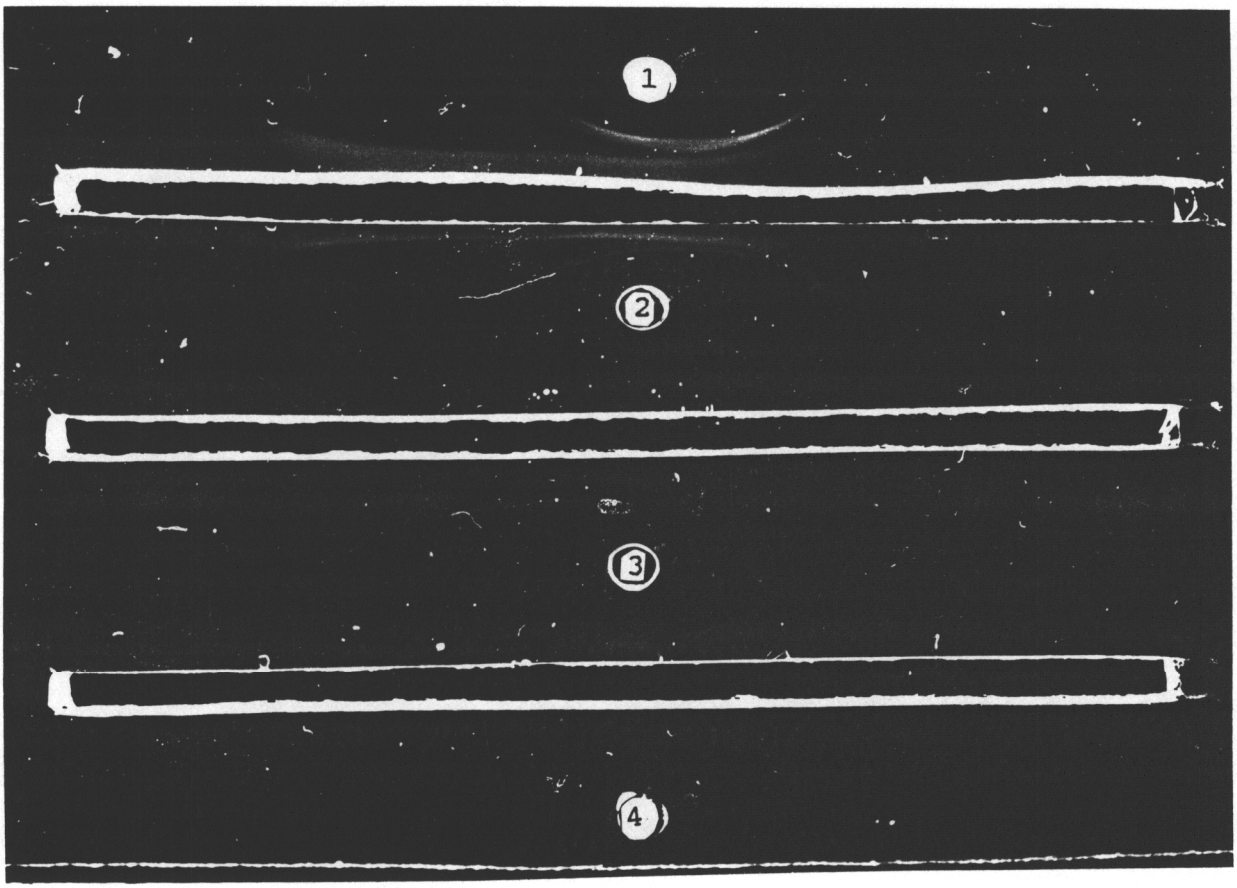

Fig. 7: Precipitation bands formed between goat antisera and antigen (fish pre- and post-immunization serum). Well 1, pre-immunization rock bass serum; well 2, 3, and 4, post-immunization rock bass, goldfish and carp serum, respectively. Trough 1, goat anti-rock bass serum; trough 2, goat anti-carp serum, and trough 3, goat anti-goldfish serum.

Table 3. Summary of the precipitation reaction of goat antiserum to fish antibodies in IE.

	Fish antibodies		
Goat antiserum	Goldfish	Rock bass	Carp
Anti-goldfish	++	+	+
Anti-carp	+	+	++
Anti-rock bass	+	++	+

VI. Elution of fish antibodies from the tube agglutination of fish antiserum with St 19

In order to obtain relatively pure fish antibodies, a solution of glycine-HCl elution buffer, pH 2.8 was employed to separate the antibodies from the antigen-antibody complex in the tube agglutination test. The antigen-antibody precipitate was treated with the elution buffer as short a time as possible to avoid damaging the antibodies by the acidic solution. Testing of the eluted antibodies with the standard tube agglutination assay demonstrated that this method was capable of separating the antibodies from the antigen-antibody complex since the eluted antibodies were able to agglutinate St 19. Eluted antibodies reached titers of up to 1:100 in the standard tube agglutination test.

VII. SDS-PADGE and Coomassie Blue stain of the eluted fish antibodies

Unlike mammalian antibodies which consist of five separate components (immunoglobulin M ,(IgM), IgG, IgA, IgD, and IgE), fish antibodies are composed of only an IgM-like immunoglobulin. The tertiary structure has a total molecular weight of approximately 800-900 kd, with heavy and light chains usually around 96,000 and 26,000, respectively. Coomassie Blue staining showed the component of fish Ig from the eluted Brucella-specific fish antibodies as two bands with a molecular weight of 90 and 27 kd, representing the heavy chain and light chain, respectively, of the fish Ig (Fig. 8). The heavy chain of eluted goldfish antibody is slightly higher, around 92 kd.

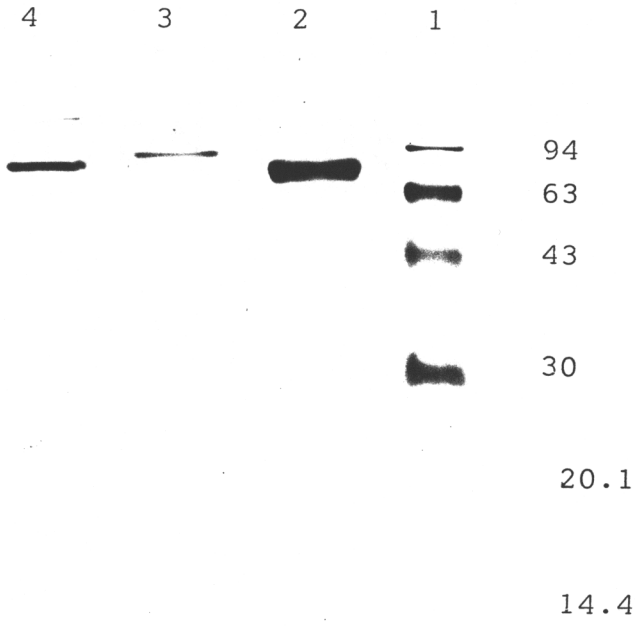

Fig. 8: Coomassie Blue stain of a SDS-PAGE of fish Ig eluted from the standard tube agglutination antigen.
Lane 1, molecular weight (kd) standard; lane 2, eluted rock bass antibody; lane 3, eluted goldfish antibody; and lane 4, eluted carp antibody.

VIII. Demonstration of Protein A's ability to bind fish immunoglobulin (Ig)

Protein A of Staphylococcus aureus has the ability to bind mammalian IgG (Surolia et al.1982). This experiment attempted to determine whether or not Protein A has the same ability to recognize fish Ig.

Using fish antibodies eluted from the STA antigen as the primary reagent, it was shown that Protein A bound fish Ig. Western Blots using Protein A exhibited two bands corresponding to the heavy chain and light chain positions of the eluted fish antibodies with all 3 fish species, rock bass, goldfish, and carp (Fig. 8). Because the goats were immunized with a complex of antibodies and St 19, they should have produced two specific kinds of antibodies, one against fish immunoglobulin and another against strain 19, particularly the O-side chain of the LPS molecule. Using LPS from strain 2308 as the antigen in Western Blot assays, Protein A recognized the specific antibodies developed by the fish (Fig. 9). Protein A was able to reveal the fish antibodies against LPS from all 3 species (Fig.10, lanes 2, 3 and 4). The same was true for the goat anti-fish sera (lanes 5-13).

The same results were obtained using eluted antibodies (Fig. 11). The results demonstrated that Protein A could bind Ig contained in the antisera from rock bass,goldfish and carp.

However, results do not show that Protein A can also bind Ig from other fish species. In order to determine whether Protein A can recognize antibodies from different fish species, different antibodies from other fish were used such as antibodies to A. salmonicida from hybrid striped bass, purified Ig from tilapia species and positive antisera containing Ig from hybrid striped bass. The results of the Western Blot, in fact, demonstrated that Protein A can bind antibodies from other fish species (Fig. 12). In order to compare the sensitivities between Protein A-HRPO and the positive antiserum against A.salmonicida, SDS-PAGE gels were loaded with A.salmonicida antigens. After electrophoresis, the protein was transferred onto nitrocellulose membranes, and the membranes were reacted with positive and negative serum, respectively. Then one group membrane of positive and negative serum was reacted with Protein A-HRPO, another group was reacted with primary rabbit anti-fish antibody-HRPO, and then with secondary goat anti-rabbit antibody-HRPO. Western Blot tests indicated that Protein A has a similar sensitivity to binding the antibody against A.salmonicida compared with the positive antisera (Fig. 13).

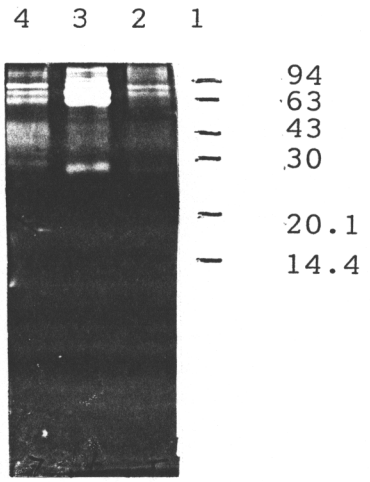

Fig. 9: Binding of Protein A to antibodies of rock bass, goldfish, and carp antiserum eluted from the STA antigen. Lane 1, molecular weight (kd); lane 2, eluted rock bass antibody; lane 3, eluted goldfish antibody; lane 4, eluted carp antibody; and lane 5, affinity-purified mouse monoclonal antibody.

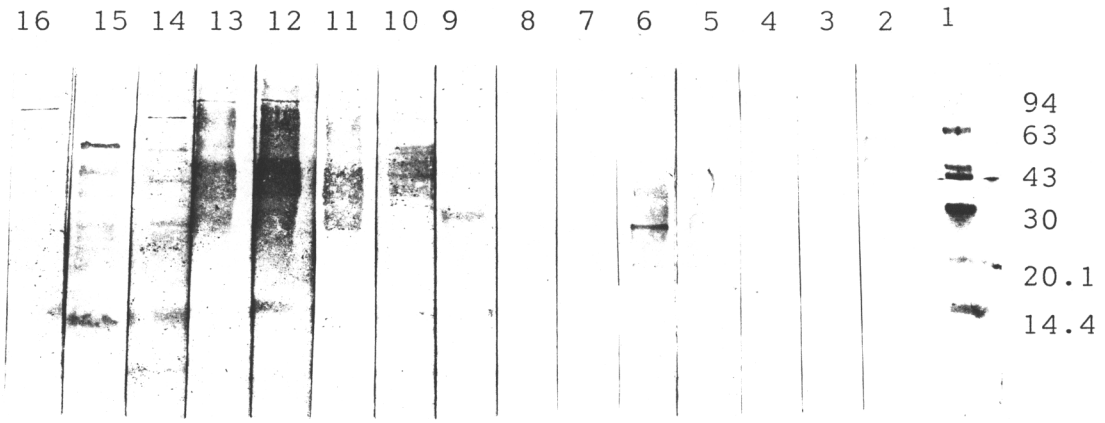

Fig. 10: Western Blots of electrophoresed St 2308 LPS developed with various reagents.

Lane 1, molecular weight (kd); primary antibodies: lane 2, 5, 8, 11, and 14, rock bass antiserum; lane 3, 6, 9, 12,and 15, goldfish antiserum; lane 4, 7, 10, 13, and 16, carp antiserum. Secondary antibodies or reagents; lane 2, 3, and 4, protein A-HRPO; lane 5, 6, and 7, goat anti-rock bass serum; lane 8, 9, and 10, goat anti-goldfish serum; lane 11, 12, and 13, goat anti-carp serum. Tertiary antibody: rabbit anti-goat serum-HRPO for lines 5-13. Primary antibody; rock bass for lane 14, goldfish for lane 15, and carp for lane 16. The secondary antibody: rabbit anti-whole fish antibody and the tertiary antibody: goat anti-rabbit serum-HRPO.

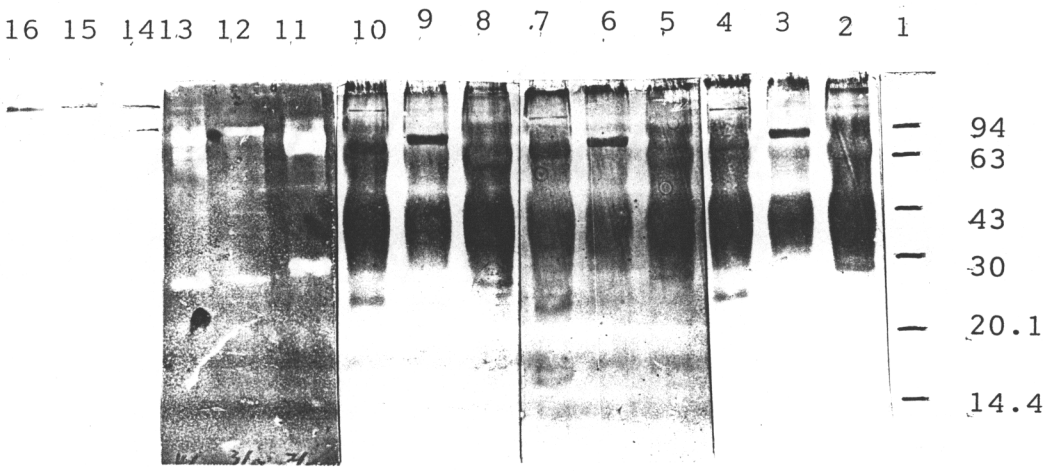

Fig. 11: Three comparative tests using Western Blot for identification of eluted antibodies by Protein A-HRPO, goat antisera, and rabbit anti-whole fish antibody.
Lane 1, molecular weight (kd); lanes 2, 5, 8, 11, and 14, eluted rock bass antibodies; lanes 3, 6, 9, 12 and 15, eluted goldfish antibodies; lanes 4, 7, 10, 13 and 16; eluted carp antibodies; primary antibody; goat anti-rock bass serum for lane 2, 3 and 4, goat anti-goldfish serum for lane 3, 6 and 9, goat anti-carp antiserum for lane 4, 7 and 10; secondary antibody: rabbit anti-goat-HRPO, Protein A-HRPO for lane 11, 12 and 13, rabbit anti-fish whole serum for lane 14, 15 and 16.

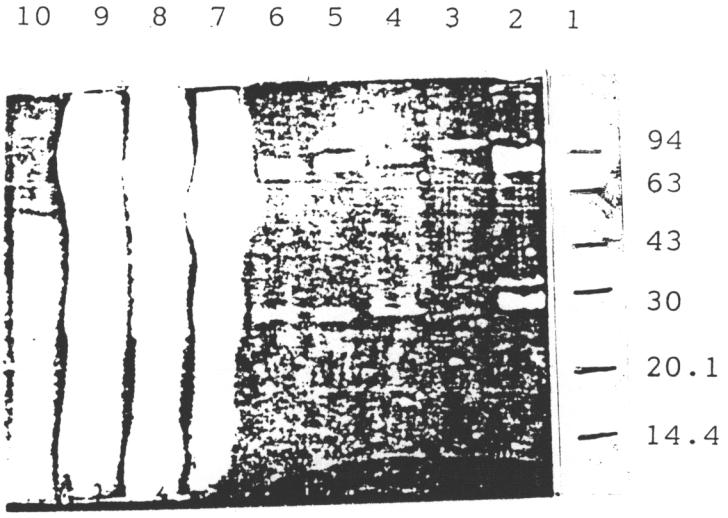

Fig. 12: The protein pattern in Western Blot for the different antisera from different fish species recognized by Protein A. Lane 1, molecular weight (kd); lane 2, rock bass serum; lane 3, goldfish serum, lane 4, carp serum (lane 2, 3, and 4, eluted antibodies); lane 5, affinity-purified tilapia Ig, lane 6, same Ig as lane 5, treated with 2-ME; lane 7, positive mouse monoclonal antibody; lane 8, positive antisera containing anti-A.salmonicida Ig; lane 9, same as lane 8, treated with 2-ME; lane 10, antibody against A.salmonicida LPS.

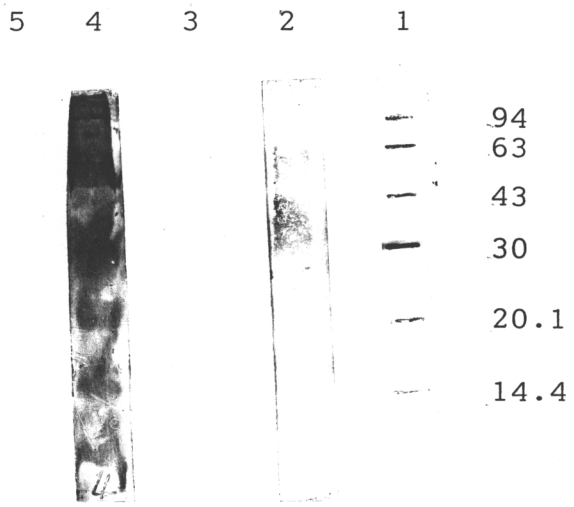

Fig. 13: Comparing the sensitivity between Protein A-HRPO and the antibody against A.salmonicida in Western Blot.
Lane 1, molecular weight (kd); lane 2 and 4, positive antiserum;lane 3 and 5,negative serum; lane 2 and 3 recognized by protein A, lane 4 and 5 recognized by primary rabbit anti-fish antibody-HRPO, and then by the secondary goat anti-rabbit antibody-HRPO.

All experiments demonstrated that the goat anti-fish sera and Protein A can bind fish antibodies to Brucella St 19, but it is unknown whether these anti-antibodies can bind the fish antibody for glochidia. In order to demonstrate that Protein A and goat anti-fish serum can recognize positive antibodies to glochidia, the antigen of glochidia was used in Western Blot tests. The results demonstrated that Protein A and goat anti-fish serum can bind the fish anti-glochidia antibody (Fig. 14), and that these immune reagents may be useful to identify the host-fish species for glochidia of mussels.

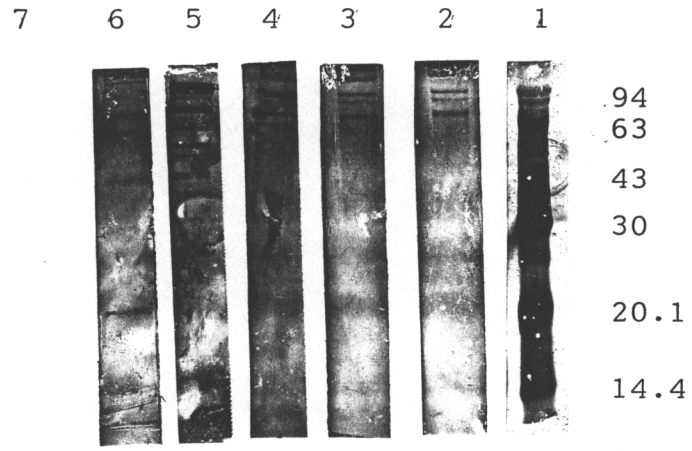

Fig. 14: Protein A, goat anti-fish serum and rabbit anti-fish whole serum detecting positive antibody against glochidia. Lane 1, molecular weight (kd); lane 2, primary antibody goat anti-rock bass; lane 3, goat anti-goldfish; lane 4, goat anti-carp; secondary antibody; rabbit anti-goat-HRPO antibody for lane 2, 3 and 4; lane 5, Protein A-HRPO; lane 6, rabbit anti-fish whole serum-HRPO.

IX. The cryopreservation of glochidia of freshwater mussels.

There are two main impediments to the cryopreservation of glochidia of freshwater mussels; 1) the embryo has shells which can prevent water flux and the uptake of cryoprotectants into the embryos, and 2) the embryos are very sensitive to subzero temperatures. In order to overcome the first obstacle, the embryos were extracted with isopropanol and hexane so that the cryoprotectants and water may permeabilize embryos. In order to overcome the second obstacle, a BD. 20 vitrification solution was used according to Steponkus (1990). A intracellular glass (vitrify) could be formed, reducing injury during chilling at subzero temperatures rather than crystal formation. The percent viability of glochidia after storage in liquid nitrogen is shown in Table 4.

Table 4. The percent viability of glochidia after storage in liquid nitrogen; glochidia were thawed and placed in an 199 media cultured at 22°C for 24 h.

Species of embryos	Days	Maximum viability (%) 1	2	3	4	6	7	8
Villosa iris		56	38	29	17	9	5	< 5

Discussion

Design of an immunological model

Parasitism is an obligate process for glochidia. They attach to the gills or fins of host fish species (Meyers et al. 1977). The glochidia obtain nutrition from the blood, transform to juveniles, and drop from their host fish to begin their free-living stage (Isom and Hudson, 1984). Once attachment of glochidia to the gills or fins of a host fish occurs, an immunological response by the host fish to the glochidia begins (Reuling, 1919; Arey, 1932; Zale and Neves, 1982; Bauer, 1987). The antibodies that the host fish produces to the glochidia of freshwater mussels may provide a serological method that can be used to identify host species (Isom and Hudson, 1982, 1984). It has been reported that glochidia of some freshwater mussels only infest one specific fish species (Meyers and Millemann, 1977; Meyers et al. 1980; Zale and Neves, 1982; Kat, 1984; Neves et al. 1985), whereas glochidia of other freshwater mussels can attach to several fish species (Tompa, 1979; Trdan and Hoeh, 1982; Isom and Hudson, 1984). Although host fish for the various glochidia are usually different, identification of hosts is important in order to preserve federal endangered and threatened freshwater

mussels.

The resistance to glochidia in non-host fish species is a non-specific immune response. Specific antibodies are developed in the host fish species in response to the glochidial infestation. So many fish species exist in nature that it is impossible to produce specific antibodies in the laboratory to each of these specific immunoglobulins to be able to identify all hosts. The question, therefore, is whether one specific reagent can detect antibodies from any fish species. Depending on the immunological reactions of host fish species to the glochidia, it may be feasible to develop such an assay.

In order to achieve such a goal, the general immune responses of three species of fish, and killed B.abortus strain 19 organisms were used to immunize host and non-host fish species, with the assumption that the LPS of B.abortus would elicit a strong immune response. The antibodies developed in the fishes against B.abortus St 19 were then used to immunize a second group of animals genetically different from the fish to induce a strong immune response. Goats were selected for this purpose in which the specific secondary antibodies to fish antibodies were produced. Such secondary antibodies may be used to recognize the fish antibodies from different fish species by cross-reactions. If the goat antibodies could recognize all fish antibodies due to cross-

reactivity, the purpose of this study would have been successfully achieved. An assay which demands less reagent preparation could be developed using Protein A which is an anti-antibody; thus, Protein A was tested for its ability to bind fish antibodies.

The humoral immune responses to B. abortus St 19 by host and non-host fish species

Previous studies (Finstad and Good, 1964; Pollara, 1970; Buhrman, 1989; Schurig, 1991) suggested that the LPS of B.abortus can evoke a strong humoral and cell-mediated immune response in both mammals and lower vertebrates. In this study, the host-fish species, rock bass, and non-host fish species, goldfish and carp, were immunized, with 3.2 X 10^5 B.abortus St 19 organisms. The tube agglutination test showed that the antibody titers of these fish species were low (1:25) after the first immunization. These titers rose rapidly to 1:800 in both host fish and non-host fish species after the second immunization, and higher titers of antibodies were achieved after the third injection. The titers reached levels of 1:6400 for host-fish species, and 1:3200 for non-host fish species. These results indicated that: 1.) the acetone-killed B.abortus St 19 can induce strong humoral immune

responses in the fish species tested; 2.) both host fish species and non-host fish species respond with similar titers to B. abortus St 19; 3.) the host fish species tended to reach higher titers of 1:6400 versus 1:3200 in non-host fish species; and 4.) all fish species tested express humoral immune responses with memory since faster and higher titers were developed after the second injection. In previous experiments, titers of 1:2560 were reached (Finstad and Good, 1964); these studies used more organisms (1×10^9 or 1×10^7) than my study (3.2×10^5).

Western Blot analysis of commercial anti-fish whole serum produced in rabbit recognized fish antibodies against B. abortus St 19 and indicated that the antibodies developed in the fishes were anti-Brucella LPS, based on the reaction position. The bands in the Western Blots corresponded to those in the earlier studies of previous investigators (Lobb and Clem, 1981; Lobb, 1986, b; Lobb and Mark, 1988). The antibodies against B.abortus St 19 can be detected by both tube agglutination and Western Blots. Similar results were observed with the Coomassie Blue test, where two bands representing heavy and light chain appeared. The most important aspect is that the tube agglutination test measures the immunological activity of fish to B. abortus St 19. The Western Blots and Coomassie Blue stains exhibited reactions given by fish antibodies (Lobb, 1986; Lobb and Mark, 1988).

These experiments suggest that the immunological reactions to Brucella between host fish and non-host fish species are not significantly different.

Goat immune responses to fish antibodies and to St 19

After specific antibodies to B. abortus St 19 were obtained from fish, goats were immunized with host fish and non-host fish antibodies agglutinated with standard B. abortus St 19 antigen. In this way, goats would be immunized with fish antibodies and St 19. Using a 1:50 dilution of fish antibodies agglutinated with B. abortus St 19 minimizes the effects of non-specific agglutinins, allowing the goats to develop specific antibodies to fish immunoglobulins. The tube agglutination test was used to measure the immunological response of the goats to the B. abortus St 19 antigen, and Western Blots measured the specificity of the goat immune response to the fish immunoglobulins.

The tube agglutination test indicated that the goats produced low levels of antibodies to B. abortus St 19 after the first immunization. The titers rose after each sequential immunization as expected (Wood and Matthews, 1987; Young et al., 1987). After the second inoculation, the titer reached was 1:400 for all goats, while after the third and fourth

immunization, the titer rose to 1:3200 in the goat anti-host fish. The tube agglutination test demonstrated that the immune response of goats to the antigen-antibody complex of fish antibody and B. abortus St 19 was capable of producing specific antibodies to the Brucella antigen and that memory existed. The use of 1:25 agglutination of antigen-antibody complex as the antigen in Western Blots also demonstrated strong reactions with goat antisera to the complex. Cross-reactions could be observed, since goat antiserum was able to react with the fish serum they were immunized with (homologous reactions) and with the heterologous fish sera.

In contrast to the tube agglutination test which only demonstrated the ability of the goat serum to react with B. abortus St 19, the Western Blot showed the ability of the goat antisera to bind fish antibody and B. abortus St 19 antigen. Because of this reaction to both antigens (fish immunoglobulin and St 19) it was not clear which reaction represented response to fish antibodies versus B.abortus St 19. In order to clarify this important point, relatively pure fish antibodies against B.abortus St 19 were required in the Western Blot test to see whether the goat antisera could bind heterologous and/or homologous fish immunoglobulins. Using this approach, cross-reaction could be detected among all three goat sera and the three fish species. This important observation also indicated that the goat antisera had

antibodies specific for fish IgM-like immunoglobulins, since the two bands representing the heavy chain and light chain characteristic of fish IgM were visible (Lobb, 1981, 1986; Lobb and Mark, 1988; Rosenshein, 1986). Due to the extensive cross-reactivity observed, it seems that the goat antisera against fish antibodies could be applied to identify the host fish species for the glochidia of freshwater mussels if this observation would hold for immunoglobulins of other fish species. The fish antibody consists of IgM (Lobb, 1981, 1986; Lobb and Mark, 1988; Litman, et al. 1990; Marchalonis, 1990) with two heavy and light chains; the anti-fish antisera always reacted with both chains no matter what fish species was tested.

Immunoelectrophoresis(IEP)

A traditional serological method, immunoelectrophoresis, has been used for diagnostic purposes in mammals and in lower vertebrates for a long time (Anderson and Klontz, 1970; Lobb, 1981; Dooley, Lallier and Trust, 1986; Grayson, 1987; Hodgins, Ridgway and Utter, 1965; Kat, 1983, Ohtani and Kawai, 1981). Finstad et al.(1964) analyzed the immunoelectrophoretic patterns of the immunological responses of the sea lamprey (Petromyzon marinus) to several antigen stimulations such as

killed Brucella abortus cells, hemocyanin, bovine serum albumin(BSA), and bacteriophage T2 (Escherichia coli). They found the development of precipitating bands between the positive sera and antigens. These bands appeared close to the wells and revealed a small amount of gamma globulin in normal adult sea lampreys. They could not decide whether the gamma globulin was immunoglobulin. Hodgins et al.(1965) analyzed electrophoretic mobility of an immunoglobulin obtained from rainbow trout serum, and the same investigator (1967) measured the nature of antibodies and the immune response in rainbow trout (Onchorhynchus mykiss) by immunoelectrophoresis. Pollara et al.(1970) detected the antibody to human "O" cells and properties of the immunoglobulin in lamprey. Anderson et al.(1970) demonstrated the precipitating antibodies against Aeromonas salmonicida in sera of inbred albino rainbow trout (Onchorhynchus mykiss). These investigations demonstrated that precipitating antibodies are found in fish antisera and that the natural precipitating antibodies are present in normal sera.

Based on these experiments, the immunoelectrophoresis assay was used in my study to determine whether this method was able to measure the immune reactions between goat antisera and antibodies against B. abortus St 19 antigen from different fish species.

Immunoelectrophoretic analysis indicated strong

precipitin lines with the goat antisera and fish immunoglobulins. In general, strong immune reactions were observed between the goat antisera and its homologous antigen, whereas weaker immune reactions were observed between goat antisera and its heterologous antigen. Among the weak immune precipitating reactions, slightly stronger reactions were observed between the goat anti-goldfish and goat anti-carp antiserum with carp and goldfish immunoglobulins, respectively, than with the rock bass immunoglobulin. All the goat antisera demonstrated cross-reactions with the immunoglobulins from the three fish species; that is, the strong reaction with the heavy background and the weak reaction with light background. However, two clear bands were always observed; one of the two bands was short and close to the well, whereas the other was long and further away from the well. The short band represents the 19s-like molecule and the long one represents the 7s molecule (Hodgins et al., 1965; Kat, 1983).

Eluted fish antibodies

Fish antibody consists of only IgM-like molecules, with a heavy chain molecular weight of about 70,000 daltons, and the light chain molecular weight of about 25,000 daltons. The

host-fish species, rock bass, and the non-host fish species, goldfish and carp, were immunized with killed B. abortus St 19 in this study. It was not known whether these fish would respond to this antigen with an antibody response consisting of IgM-like molecules. In order to demonstrate this aspect, fish antibodies were eluted from agglutinated St 19 organisms to obtain relatively pure fish antibodies and to verify that the fish immune responses to killed B. abortus St 19 is fish IgM, as opposed to other fish immune responses. The eluted specific fish antibodies were injected into goats to produce specific antibodies to the fish IgM. Because antigen-antibody complexes can be separated under acidic conditions, glycine-HCl elution buffer, pH 2.8 was used. In order to avoid damage to the specific antibodies by the acidic solutions, the antigen-antibody complexes were treated with this solution as briefly as possible. Testing of eluted antibodies in tube agglutination demonstrated that this treatment was able to separate the fish antibodies from the antigen-antibody complex, and that the eluted antibodies were still functional.

The SDS-PAGE pattern of the eluted fish antibodies, as visualized by Coomassie Blue Stain, consisted of two bands corresponding approximately to 70,000 Kd and 26,000 Kd which represent the heavy and light chains of fish IgM. Taken together, these results indicate that 1.) the goat humoral immune response to the fish antibodies is a specific anti-

fish IgM response; 2.) that two bands, one short and one long, appeared in the immunoelectrophoresis and could be considered to be the 19s and 7s form of fish immunoglobulins; and 3.) that the antibodies of rock bass, goldfish and carp are IgM-like.

Protein A binding to fish immunoglobulin (Ig)

Two assays, Western Blot and immunoelectrophoresis, demonstrated that the secondary goat antisera can recognize the first antibodies of all three fish species,rock bass, goldfish and carp. Western Blots and immunoelectrophoresis also exhibited the capability of cross-reactions; that is, one specific goat antiserum can react with the antibodies of all three fish species. Although the goat antisera can bind with the fish antibodies, the processes to produce these reagents appear costly and time consuming. It is necessary to find a reagent which replaces the goat antiserum so that the manipulation may be made simpler and more consistent. A good candidate is Protein A since it is an anti-antibody and can bind various antibodies from mammals (Surolia et al.1982), and is commercially available. There appears to be no literature available indicating that Protein A has been utilized directly to detect fish immunoglobulins.

Hastings et al.(1988) used Protein A from Staphylococcus aureus to detect rabbit anti-trout IgM (RaT IgM) reacting with trout serum to Aeromonas. Cook et al.(1991) used Protein A gold as a marker to demonstrate gonadotropin releasing-hormone receptors on gonadotrophs and somatotrophs of the goldfish in electron microscope studies.

If Protein A can directly bind fish antibodies, it can be applied to identify host-fish species for glochidia. Results observed with Western Blots showed that Protein A can bind all three fish antibodies eluted from the antigen-antibody complex of fish sera agglutinated with St 19 antigen. The Western Blot patterns indicated reaction with two bands, the heavy chain and light chain of fish IgM. It also showed that the Protein A pattern was similar to the one observed with the goat antibodies.

In my study, the killed B. abortus St 19 that contained the major LPS antigen were exposed to the three fish species. The studies demonstrated that Protein A could detect the presence of specific fish antibodies against the LPS antigen from Brucella. In order to demonstrate this, three comparative Western Blot tests were carried out with Protein A, goat antisera and rabbit anti-whole fish serum. The results indicated that Protein A can bind the specific antibodies against Brucella LPS, and the binding capability of Protein A appeared to be similar to the one displayed by the

goat antisera and rabbit anti-whole fish serum. Ideally, if Protein A could bind the antibodies of fish species against different antigens, it could be applied for detecting different fish diseases in the clinic. Based on this potential use, two immune sera from other fish species against antigens different from Brucella were used to see whether Protein A can bind these antibodies. The results in Western Blot indicated that Protein A could do this by binding to antibodies of rock bass, goldfish and carp, affinity-purified tilapia Ig, tilapia Ig treated with 2-ME, and positive antisera containing anti-A.salmonicida Ig, antisera. Protein A appears to react with the antigen similar to the positive antisera against A.salmonicida.

Protein A has not been reported in the literature as binding fish Ig. In this study , Protein A can bind various fish Ig's. Therefore Protein A could be used for the purpose of identifying the host fish species for the glochidia of freshwater mussels and may also be a powerful tool for the diagnosis of fish diseases in the clinic.

Measuring positive fish antibodies against glochidia

This study was carried out to develop an immunological test which could identify host fish species for glochidia of

freshwater mussels. Various experiments were designed to demonstrate that goat antisera made against one fish species as well as Protein A can recognize various fish antibodies. The use of Protein A-HRPO in the assay tended to be a more convenient, reliable, sensitive, quick and simple method for this purpose. The final goal of this study was to develop an immunological method to identify host fish species of glochidia. Although the described experiments showed that goat antisera and Protein A are good reagents to detect fish antibodies from different fish species, it is not clear whether these reagents could detect fish antibodies against glochidia.

Using three different antisera; goat antisera, protein A and rabbit anti-fish whole serum, Western blot tests suggested that all of them can bind antibodies from host-fish species specific for glochidia of freshwater mussels. The results indicate that both goat antisera and Protein A may be useful in a serological method to identify host fish species of glochidia.

Cryopreservation for glochidia of freshwater mussels

The glochidia of freshwater mussels were treated with isopropanol and hexane to change the permeability of the

shells of the mussel glochidia allowing the water flux and the uptake of cryoprotectants into the glochidia which is precluded without this procedure. Conventional cryopreservation procedures do not prevent intracellular ice formation that damages the glochidia during the freezing period. The use of BD.20 vitrification solution precludes intracellular ice formation and the glochidia can be cooled and warmed at ultra-rapid rates minimizing chilling injury and permit the recovery viable glochidia following storage in liquid nitrogen. The assay used in this study for cryopreservation of mussel glochidia is superior to conventional cryopreservation method since it permits more glochidia to survive for a long period of time. Nevertheless, less than 1% of the thawed glochidia were alive 8 days after thawing indicating that further improvements are needed to allow long time survival.

Conclusions

1. All experiments in this study indicated that it may be feasible to establish a serological method capable of identifying host fish species for glochidia of freshwater mussels.

2. The Brucella abortus strain 19 LPS evoked a humoral immune response in three fish species (rock bass, goldfish and carp).

3. The titers of tube agglutination tests for fish antisera against St 19 indicated that such immune reactions can show memory.

4. Goats can produce a strong humoral immune response to the antigen-antibody complex of fish antibody and St 19 antigen. The immune response to the antigen-antibody complex has memory.

5. The Western Blot test demonstrated that goat anti-fish serum can cross-react with all fish antibodies used in this study.

6. The specific fish antibodies eluted from STA antigen consist of fish IgM-like molecules with heavy and light chain components.

7. Protein A can recognize fish antibodies, reacting with both the heavy and light chain of fish Ig.

8. Protein A can bind specific fish antibodies against B.abortus St 19, as well as bind antibodies directed against

A.salmonicida including glochidial antigens.

9. It is feasible to use Protein A as a serological tool to diagnose fish diseases.

10. viable glochidia of freshwater mussels can be maintained for 5-6 days after cryopreserving (using a vitrification in protocol) and thawing them..

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