IMMUNOFLUORESCENT TECHNIQUES APPLICABLE TO DETECTING AEROMONAS LIQUEFACIENS

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ABSTRACT

The use of fluorescent labelled antibody (FA) systems provide a means for detecting both the presence of the agent, *Aeromonas liquefaciens* and fish antibodies to that agent. Bacteria from experimentally infected fish could be identified within 6 hours after obtaining the sample by the FA technique.

The preparation of specific antiserum to *A. liquefaciens* and a proposed method for rapid detection of the bacterium from field specimens is presented.

INTRODUCTION

Aeromonas liquefaciens is responsible for considerable losses in many warm water fisheries throughout the world. In spite of this, very little information exists on the host range and natural distribution of the pathogenic *A. liquefaciens*. Such information would be useful in constructing guidelines for control of diseases due to this agent.

Studies on the epizootiology of various disease agents are often based both upon cultural and serological procedures for establishing the identity and distribution of the particular agent. While cultural identification of *A. liquefaciens* is comparatively simple, cultural procedures are time consuming and disease episodes can often progress beyond the point for effective control by the time the agent is isolated and identified. Furthermore, if diseases due to this agent are to be controlled, techniques must be developed which provide for the screening of large numbers of hosts. Preferably those procedures would provide for the detection of both the agent and host antibodies to the agent if such antibodies develop. Immunofluorescence procedures would appear to fulfill those requirements, thus an effort was directed toward investigating immunofluorescent techniques for detecting *A. liquefaciens*.

MATERIALS AND METHODS

Anti-A. liquefaciens serum. Polyvalent *A. liquefaciens* antiserum was prepared from strain nos. MS 337, MS 338, MS 339, MS 340 and MS 350 by a procedure described elsewhere (2). Briefly that procedure is as follows. Two plates of brain heart infusion agar were inoculated with each organism and the plates incubated at 20C for 48 hours. The cells were harvested into saline, the suspensions pooled and boiled for 2.5 hours. The pooled suspension was washed 2x by centrifugation and resuspended in saline to yield a suspension approximately equal to a No. 2 MacFarland Nephelometer tube. The animals were injected intravenously with 0.25 ml, 0.5 ml, 1.0 ml and 2.0 ml of the preparations at five day intervals. Eight to ten days after the last injection, the animals were exsanguinated, the serum collected, thimerosal added to a final concentration of 1:10,000, and the serum conjugated as described below.

Anti-Fish globulin serum. A modification of Proom's method (3) was used to prepare antiglobulin serum to plasmas of Fathead minnows *(Pimephales promelas)*, Golden shiners, *(Notemigonus erysoleucas)* and the Gulf Killifish *(Fundulus similus)*. Caudal fins of the fish were severed and the blood collected in heparinized capillary tubes (100mm long and 1.2 mm I.D.). The tubes were sealed on one end and centrifuged in a clinical hematocrit centrifuge. The tubes were then broken at the erythrocyte-plasma interface and the plasma portions pooled. The plasma was diluted 1.0 part plasma to 3.2 parts distilled water. To this mixture was added 0.86 parts 10% aluminum potassium sulfate and the pH was

adjusted to 6.5 with 5N sodium hydroxide at which point a turbid suspension developed. The suspension was centrifuged and washed 2X in saline containing 1:10,000 theimerosal. After the second wash, sufficient saline was added to make the final volume of the mixture equivalent to four times the amount of the original plasma. Five ml of the suspension were injected into each hind leg of the rabbits. Ten to 14 days after the last injection, the rabbits were exsanguinated and the sera conjugated with fluorescein isothiocyanata as described below.

Conjugating Rabbit Antisera. Rabbit globulins were precipitated with ammonium sulfate in a manner similar to that described by D'Aessio *et al.* (1). The ammonium sulfate solution was prepared by dissolving 542 g of ammonium sulfate in a liter of distilled water, cooled to 4 C and the pH adjusted to 6.3 with 5N NaOH. The ammonium sulfate solution was warmed in a water bath until the precipitated crystals dissolved, an amount equal to that of the serum added to a burette, and the solution equilibrated to ambient temperature. The ammonium sulfate was added dropwise with constant slow stirring to the plasma, then placed at 4 C and stirred for 2.5 hours. Afterwards, the preparation was centrifuged and the sediment resuspended in a minimum amount of distilled water. The preparation was desalted and equilibrated with carbonate-bicarbonate buffer pH 9.2 by passage through a Sephadex G-50 column. The amount of protein was estimated by comparing the absorbancy of the solution at 280 mu with a standard curve. The globulin solution was adjusted to 2% protein solution and fluorescein isothiocyanate powder added 20 mg powder/gm protein. The preparation was stirred overnight at 4-6 C. Afterwards, excess dye was removed and the conjugate equilibrated with phosphate buffered saline (PBS) pH 7.2 by passage through a Sephadex G-50 column.

The optimum titers of the conjugates were ascertained by reacting twofold dilutions of the conjugates with appropriate antigens. Smears of 18-24 hour broth cultures of *A. liquefaciens* were prepared for these purposes.

The anti-*A. liquefaciens* conjugate was assayed by applying the various dilutions of the conjugate to the smears, and processing them as for the direct FA test as described below.

The anti-fish conjugate was assayed for use in the indirect FA test. Plasma derived from fish which one month previously had received heat killed *A. liquefaciens* cells were diluted 1.8. The plasma preparations were applied to *A. liquefaciens* smears and incubated at ambient temperature in a humidity chamber for 45 minutes. The preparations were washed with PBS and various dilutions of the anti-fish conjugate added. The preparations were incubated at 37 C, washed in PBS and mounted in PBS glycerol.

Duplicate swabs derived from 10 experimentally infected fish and from 14 field cases were placed in ten ml brain heart infusion broth. One of each of the broth cultures was processed in the conventional manner for cultural identification after incubating the broth at 30 C overnight. The other broth culture was processed for direct FA staining after 6 hours incubation.

Direct FA test. The broth cultures were centrifuged lightly to sediment cells and the broth supernatants discarded. The cells were resuspended in two ml PBS and smears prepared. After air drying, the smears were heat fixed and the rabbit andti-*A. liquefaciens* conjugate was added. The preparations were incubated 45 minutes at 37 C. The slides were washed with PBS, blotted dry and mounted with glycerol PBS. Overnight broth cultures of *Escherichia coli, Serratia marcesens* and *A. liquefaciens* were processed in a similar manner and served as controls.

Indirect FA test. Three species of fish mentioned earlier received 0.05 ml heat killed *A. liquefaciens* cells intraperitoneally. Samples were derived at weekly intervals by pooling the bleedings of five of each species of fish. Two-fold serial dilutions of the plasma were applied to smears

of *A. liquefaciens* and incubated 45 minutes at ambient temperature. The preparations were washed in PBS, the appropriate anti-fish conjugate added and after 45 minutes incubation, the preparations were washed in PBS and mounted. In addition, smears of *E. coli* and *S. marcesens* were processed with the fish plasma to test for non-specific binding of the plasma.

RESULTS AND DISCUSSION

The optimum concentration of the anti-*A. liquefaciens* conjugate was 1:32. The optimum concentration of the various anti-fish conjugates were 1:16 for the anti-*Pimephales promelas* conjugate, 1:16 for the anti-*Notemigonus erysoleucas* conjugate and 1:8 for the anti-*Fundulus similus* conjugate.

There was perfect correlation between direct FA results and cultural results derived from the processed specimens. *A. liquefaciens* was revealed in all experimentally infected fish and in 12 of the 14 suspect field cases.

Antibody to *A. liquefaciens* was revealed in all fish by the indirect FA test. The maximum antibody response was observed approximately one month after exposing the fish to the heat killed cells.

These data suggest that immunofluorescence procedures are applicable to rapid detection of *A. liquefaciens* infections by using the direct FA test. However, until the antigenic relationships among the various *A. liquefaciens* can be more clearly defined, considerable difficulty will be encountered in distinguishing those strains capable of causing disease from those ubiquitous strains that are apparently non-pathogenic.

The indirect FA apparently provides a means for detecting antibody to *A. liquefaciens* hence previous infection with the agent. The indirect FA would appear to be ideally suited for antibody assay when small quantities of serum are available.

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INVESTIGATION OF STRIPED BASS, Morone Saxatilis (WALBAUM), CULTURE IN OKLAHOMA [1]

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ABSTRACT

Various stocking rates were tested over a three year period for production of striped bass in culture ponds. Rates ranged from 10,000 to 160,000 fry per acre. Results indicate higher rates produced as high a percent yield as lower rates.

Four food types were also tested during this period to determine which produced the best yields. Commercially prepared supplemental feeds did increase production over natural foods.

Food habits data support earlier information that Copepoda, Cladocera and Insecta are important food organisms to juvenile striped bass.

INTRODUCTION

The establishment of a land-locked striped bass, *Morone saxatilis*, (Walbaum), population in the Santee-Cooper Reservoir system of South Carolina created great interest among fishery managers nationwide. They became interested in the suitability of this species for control and utilization of large shad populations in warmwater reservoirs. In addition, the sport fishing related industry generated in association with the population attracted national attention (Stevens, 1964).

Early attempts to establish this species in other inland waters were largely unsuccessful due to unhealthy stock, hauling failures (Surber 1957, Gray 1952) and general failure of fry stockings regardless of numbers. Sandoz and Johnston (1965) suggested rearing striped bass to fingerling size before stocking to reduce early mortality experienced with fry stockings.

Attempts were made to rear fingerling striped bass in Oklahoma in 1965 and 1966 but results varied. A research project (DJ-F-25-R) was

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