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Fish Disease Inspection and Certification

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ABSTRACT—Many countries now require that incoming shipments of salmonid fishes be inspected and certified free of certain diseases. This paper briefly describes general procedures for conducting disease inspections of salmonid rearing facilities. In addition, methods for detecting the presence of bacterial pathogens, the sporozoan Myxosoma cerebralis, *and three viruses (infectious pancreatic necrosis, infectious hematopoietic necrosis, and viral hemorrhagic septicemia) are outlined. Modifications of inspection procedures are also discussed.*

The growing awareness of the spread of fish diseases by indiscriminate transfers of fish has led to the development of laws and regulations controlling the importation of fish, fish eggs, and some fish products in at least 42 countries. Some countries, particularly the United States, Canada, and a number of others, require that incoming salmonid fish shipments or the hatcheries of origin be examined by a recognized hatchery inspector and certified free of certain diseases. In addition, some U.S. States and Canadian Provinces require that fish shipments imported from other states or provinces within the country also be inspected.

Most of the inspection and diagnostic procedures which I will describe pertain only to salmonid diseases. The methods for the most part are those which were approved by the Fish Health Section of the American Fisheries Society (1975), and are now available as a manual. Therefore, I will not go into each procedure in detail; instead, I will concentrate on some of the problems that I have encountered when using these procedures or some of the changes which I have made to fit certain needs.

DISEASE-FREE CERTIFICATION OF FISH

For a fish rearing facility to be certified free of a specific disease or diseases, periodic inspections must be conducted by a qualified biologist. Frequency of inspections and the number of inspections required before disease-free certification can be issued varies from nation to nation, but inspections are usually conducted once or twice yearly with two to four samplings required prior to issuance of disease-free certification. Issuance of this certification is also based on the hatchery owner's warranty that no uncertified fish or eggs have been brought to the hatchery subsequent to inspection and no major undiagnosed mortalities have occurred since inspection.

During an inspection, it is imperative that all lots of fish present at the facility be sampled by the certifying inspector. A lot is defined as fish of the same age which have always shared the same water supply and which have originated from a discreet spawning population.

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Sampling

After determining the number of lots in a hatchery, the next step is to decide on the number of fish to be sampled from each lot. In an inspection, the hatchery inspector is attempting to detect asymptomatic carriers as well as diseased fish. Therefore, the method of determining the actual number of fish to be sampled is based on obtaining a 95 percent probability of detecting at least one disease agent carrier in a lot with an assumed prevalence of carriers. According to American Fisheries Society procedures, the assumed carrier incidence is 5 percent for all diseases which are detectable in the carrier state except infectious hematopoetic necrosis (IHN) virus where the assumed incidence is 2 percent. Some other countries have slightly different procedures based on different assumed carrier incidences. Tables are available to help the inspector determine the correct sample size for each lot of fish. Once the correct sample size has been determined, the inspector must follow approved procedures for selecting tissues to be sampled and for processing the sample tissues. The procedure, used, of course, depends on the disease agent or agents which the inspector is trying to detect.

Viral Diseases

The pathogens which I have most commonly inspected for are the three salmonid virus disease agents: viral hemorrhagic septicemia (VHS), infectious pancreatic necrosis (IPN), and IHN. At present, the only approved procedure for determining the presence or absence of any of the viruses involves the isolation of the virus from fish tissues or sex products on cell culture. For detection of IPN and VHS viruses, I sample the posterior kidney and spleen from fish over 5 cm in length, entire viscera from fingerlings up to 5 cm, and for sac fry, the entire fish. For IHN virus, the only reliable way to detect the carrier state is to sample the ovarian or seminal fluid of spawning fish. Ovarian fluid is preferred for most fish species except for rainbow trout where seminal fluid has been found to carry the virus to an equal extent. Samples for virological analysis are transported to the laboratory on ice,

but are never frozen, because some IPN virus strains lose viability after freezing and thawing. Visceral samples are processed by first homogenizing in saline and then centrifuging the homogenate to remove debris and bacteria. Total dilution of visceral samples prior to inoculation should not exceed 1:200. The ovarian fluid samples are usually processed like the tissue samples, except that they are preferably not diluted, or at the most not more than 1:10.

Samples are then decontaminated by adding penicillin, streptomycin, and mycostatin. Gentamicin[1] may be substituted for the penicillin and streptomycin and it may be preferable since it inhibits some of the mycoplasmas and is more efficacious against some of the pseudomonads. Pseudomonads sometimes give a false cytopathic effect (CPE) reaction which resembles that of IHN. After a 2-hour period at 15°-20°C to allow the antibiotics and antimycotics to work, the samples are refrigerated until inoculation. The samples must be inoculated into cell cultures within 7 days after they are collected.

I use the rainbow trout gonad (RTG-2) cultures for IPN detection, and fat head minnow (FHM) cultures for IHN and VHS detection. Some inspectors use only one cell line for all viruses, which is acceptable, but I use two lines because I have found that the RTG-2 line is much more sensitive to IPN virus and the fat head minnow line seems to be a little more sensitive to the IHN virus. In addition, the use of two lines in many cases for one sample gives a double chance for virus isolation, particularly if something should go wrong with one set of cultures. Both of these cell lines are stable, have been karyotyped, and are commercially available. All of my cell cultures are grown in Eagle's MEM with Earle's base and with 10 percent agamma newborn calf serum added. Fetal calf serum may be used with good results. I use the new Falcon Multiwell Plate or Linbro Multi-Dish Tray rather than conventional tissue culture tubes. Each of the 24 wells in the plates easily hold about 1 ml of media, but the plates occupy much less incubator space and are quicker to inoculate and read than are tubes.

The plate may be handled as a closed system by using an adhesive film to cover the wells, or as an open system by using the lid which comes with the plate. To maintain the pH in the open system, I use Tris buffer. Samples are inoculated into cell cultures when the cultures are at least 24 hours old, but not more than 72 hours. When the cell monolayers are 80 to 90 percent confluent, 0.1 ml of each sample is inoculated into each of two cell cultures. Positive and negative controls are included on each culture plate or on each set of culture plates for one cell line. The inoculated cultures are incubated at 15°C for all three viruses and are observed for at least 14 days for the development of viral CPE. Material from any suspicious-looking cultures is inoculated into fresh cultures. If the CPE resembles that caused by any of the three salmonid viruses, a serum neutralization test is performed using specific antiserum to confirm the identity of the isolate. I usually use serum produced in either trout or rabbits for this purpose.

PARASITIC DISEASES

The only parasite which I have routinely inspected for is *Myxosoma cerebralis,* the causative agent of whirling disease. To detect this parasite, I use the method developed by Markiw and Wolf (1974). The inspector samples heads of fish between 4 and 12 months of age, as this is the age where the fish are still small enough to handle easily, but are old enough to be carrying spores rather than just trophozoites. The fish are processed in pools of up to 60 fish. The heads are heated for 5 to 10 minutes in water at 50°C to make flesh removal easier. The flesh, brain, spinal column are discarded, retaining the skull and gill arches. These elements are digested in acidic pepsin solution at 37°C until no chunks remain. The mixture is centrifuged at 1,200 *g* for about 10 minutes. The pellet is then further digested in basic trypsin solution at 22°C for 30 minutes. After stopping the trypsin digestion by adding serum, the material is centrifuged again and the pellet is resuspended in a small amount of saline with serum. It is layered on top of a 55 percent dextrose solution and centrifuged at 1,200 *g* for 30 minutes. If the spores are present, they will be found in a pellet at the bottom of the dextrose gradient. The efficiency of spore recovery with this method, according to Wolf, is about 80 percent.

I have found this method to be quite satisfactory for the most part, but have experienced one problem with it. I have seen some false positives in certain areas in the State of Washington where coho and chinook salmon carry a sporozoan, *Myxobolus kisutchi,* in the hind brain and spinal cord (Yasutake and Wood, 1957). The parasite looks very much like *Myxosoma cerebralis.* Although we remove as much of the brain and spinal column as we can when processing the fish, if the incidence of this parasite is very high, spores frequently will remain. There are criteria to distinguish between the two parasites. For example, *Myxobolus* has an iodinophilous vacuole, while *Myxosoma* does not. This can be determined by staining viable spores with an iodine preparation and examining microscopically. Another procedure which can be used is to examine the head and spinal cord area histologically to determine the location of the parasite. *Myxobolus kisutchi* occurs in the brain and spinal cord only, and not in the cartilaginous or bony elements of the head and spinal column where *Myxosoma cerebralis* occurs.

BACTERIAL DISEASES

I have been asked occasionally to inspect for the presence of certain bacterial diseases. If the hatchery is being inspected for certification, I usually select only moribund fish from the hatchery over a period of several months. I sample by streaking material from the kidney or other organs which exhibit lesions onto the appropriate agar medium. I also gram stain the material and look for bacterial pathogens. Isolated organisms are identified primarily by biochemical testing, since serological reagents have only recently

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

become available. Standard media are used in conjunction with the API Analytab system. This system was designed for the identification of Enterobacteriaceae and other gram-negative organisms, such as *Pseudomonas, Aeromonas, Vibrio,* and *Flavobacterium*. Since most fish pathogens are gram negative, the system works fairly well for identifying them. The API Analytab contains dehydrated media for over 20 different biochemical determinations. The media is reconstituted by adding distilled water or saline in which the colony to be identified is suspended. The colony is picked from a culture plate for this determination. This system is well suited to a small laboratory which may not be able to produce many kinds of biochemical test media. One of the main advantages of the API Analytab, besides its compact size and ease of use, is the coding system which has been developed by API for quick and easy identification of the organisms. The system is designed primarily for human pathogens, rather than fish pathogens, but a number of fish organisms, such as *P. flourescens* and *A. liquifaciens,* are included. In addition, I have modified the coding to identify other fish pathogens.

I should mention that for the bacterial kidney disease organism (*Corynebacterium* sp.), which is not very amenable to culturing, I have been relying up to this point strictly on gram stain characteristics. Recently, immunodiffusion and indirect flourescent antibody techniques have been developed to detect the presence of the kidney disease organisms in fish. Both of these methods are much more sensitive than the old gram stain method, and both are described in the American Fisheries Society (1975) disease detection procedures manual. The flourescent antibody technique is a little more suited to our needs since it takes about 1-2 hours to complete, as opposed to 20-24 hours for the immunodiffusion technique.

DIAGNOSTIC EXAMINATION OF FISH

For diagnostic purposes, as opposed to disease-free certification, 25-50 diseased fish are requested. I prefer to receive the fish while they are still alive. If this is not possible, I request that they be sent on ice, but not frozen, or that some be fixed in Bouin's solution and the rest sent on ice. When the live fish are received, they are divided into four groups. Some are examined externally and internally for parasites, some are prepared by bacteriological analysis, some are processed for virological analysis, and the rest are placed in Bouin's fixative for histological sectioning. The use of four methods of analysis provides a good picture of the disease pathology and its etiology.

MOVING TOWARD IMMUNOLOGICAL TECHNIQUES

The diagnostic and inspection field is moving more and more toward immunological techniques such as serum neutralization, immunodiffusion, immunoflourescence, and immunoperoxidase for the detection and identification of fish disease agents. These methods should help to revolutionize the field, as they are much more sensitive and rapid than many of the older techniques.

LITERATURE CITED

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