Winter School on

'RECENT ADVANCES IN DIAGNOSIS AND MANAGEMENT OF DISEASES IN MARICULTURE'

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Course Manual

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AN OVERVIEW OF DISEASES IN MARICULTURE

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The importance of aquaculture in the agricultural development of India is well known. The protein intake of our population is well below the required amount. The continued population growth in south East Asian countries depletes the available source of animal protein. Fish provide a cheap source of animal protein. Fish production in many parts of the world is stagnant because the major source of fish production comes from open waters. There is no way to improve these sources.

There is considerable scope to improve the animal protein resources by resorting to intensive and scientific methods of animal husbandry and aquaculture. Among these two, aquaculture has considerable scope for augmenting production and providing gainful employment to the weaker sections of society. In order to improve the productivity fish are maintained at high density and given artificial formulated feeds. The high density favours the spread of pathogens. All types of animal husbandry break down the natural barriers between pathogen and its potential host. Hence, disease is one of the most serious limiting factors in aquaculture. Diseases devastate the entire crop many times. The frequency and severity of disease out breaks have increased many folds with the spurt in aquaculture.

Aquatic environment

Aquatic environment is intimately associated with fish/ shellfish health. There are several parameters in aquatic environment, which determine the maintenance of homeostasis of the organism. Alteration of these parameters beyond certain limits adversely affects health of the organism. They either predispose them to the attack of pathogen or may cause disease itself. Most important parameters are temperature, the intensity and periodicity of light, the chemical composition of the water and its biological content, the availability of space, food and frequency of fright stimuli such as moving shadows.

Carbon dioxide

Carbon dioxide/ carbonate/ bicarbonate in water forms the reservoir carbon for photosynthesis by aquatic plants. This provides the basis for fish food production. This closely associates with buffering capacity. During daytime CO_2 is removed by aquatic plants, hence, the pH rises but during night plant respiration releases carbon dioxide and there will be fall in pH. The carbon dioxide level must be below 6 mg/litre. Increase in CO_2 depresses fish respiration. The minimum threshold oxygen requirement of fish increases with rise of carbon dioxide. Fish can adjust to high levels of carbon dioxide provided the change take place slowly. Fish generally avoids carbon dioxide levels as 1.6 mg/litre. Intensive culture generates high amount of carbon dioxide due to fish metabolism and microbial decomposition.

Ammonia

The un - dissociated ammonia is highly toxic. The un-dissociated ammonia occurs in alkaline pH. High protein diets fed to fish in intensive culture system result in high level of ammonia.

Hydrogen sulphide

High organic load and high protein diet result in liberation of hydrogen sulphide. It is extremely toxic. The maximum accepted level is 0.002 mg/litre.

Contamination and pollution

Water can become contaminated with harmful substances by their penetration with water intake, with run off, seepage from surrounding lands; by their formation with the ponds, as the result of normal biological processes and their production by planktonic or other organisms inhabiting the pond.

Serious pollution often results from human activity. They are domestic effluents, agricultural and industrial effluents. These include insecticides, fertilizers and harmful chemicals. The only way to protect fish is to prevent them entering aquaculture facilities.

Biotic environment

Several organisms co-exist with fish and prawns as components of the same environmental complex. They become harmful to the cultured species. These harmful effects are either direct or indirect. Activities of some organisms alter the environmental parameters in such a way effects are either direct or indirect. Activities of these organisms make the environment less capable of sustaining the cultured species. Intermediate host of fish parasites living in aquatic system is also indirectly harming the fishes. Direct effects are exemplified by the activities of insect larvae inflicting injuries that eventually kill fish larvae. Another example is that when stocking density is high and fish of various age groups are mixed they develop cannibalism.

Algae

Algal colonies can form surface scum or hair like, matted filaments suspended in water. Some algae embed in the surface tissues of fish. They may clog gills and cause asphyxia.

Several genera of blue green algae produce dangerous blooms, which will result either in algal toxicosis or disturbance in oxygen balance. These blooms occur due to excess fertilization of ponds by over enthusiastic farmers. Photosynthetic process produce excess oxygen during daytime and water will be super saturated with oxygen leading to gas bubble disease. During night algal respiration lead to oxygen deficiency and predawn death of fish occurs.

Over crowding of algae lead to lack of nutrients and mass death of algae result. Dead and decomposing algal cells release toxic products. The fish die due to toxicosis in presence adequate oxygen supply. *Microcystis aeruginosa, Peridinium sp., Gymnodinum, Spirogyra, Hydrodictyon etc.* are some of algae causing blooms. Copper sulphate is the best algicide. 0.7 ppm solution is sprayed over the pond to control the bloom. Filamentous algae can be controlled by lime treatment.

Monitoring of farms

Most workers face difficulty in diagnosing disease problem, when it arises in a farm. Early detection is the key to success. It is essential to develop knowledge of the normal condition of fish. This is necessary for the recognition of abnormalities. Therefore a record on the physiological parameters of normal health status must be maintained. Any deviation from that health standard indicates abnormality-disease. Hence, periodical monitoring and observation of production facilities shall obtain base line information. Once these information or data is obtained the frequency of visits to that site can be reduced. Detailed records are made on such visits. The managers of farm must be interviewed. The data can be broadly classified into 1. Fish 2. The environment 3. Water.

The fish

The species of fish cultured. 2. Mortality pattern-what type of fish. 3. Dead fish preserved. 4. Feeding schedule. 5. Appearance of fish. 6. Any change in routine. 7. Diet. 8. Presence of parasites in fish. 9. General condition of fish-histology hematology and microbiological status.

The environment

a) Ecology
b) Physical
c) Chemical

Ecology

1). Type, size, and location of facility. Smaller habitat-fewer parasites
2). Depth of water-artificial habitat- abundance of parasites.
3). Water supply facility.
4). Aquatic vegetation
5). Type of bottom
6). Immediate surroundings
7). Population density
8). Habitat utilization
9). Animal life
10). Recent changes

Water

Temperature – at various time intervals of day and night and at various depths
Colour-indicate the type and quantity of dissolved matter and particulate matter.
Transparency - opalescent, cloudy, transparent, opaque. Presence of precipitate.
Taste and smell-sweet, sour, bitter and salty.

Chemical analysis

pH, oxygen, hardness, carbon dioxide, total solids, iron, hydrogen sulphide, and salinity.

Bottom of pond

Chemical analysis, particulate matter and bacteriological examination.

Sampling

The selection of samples depends on the purpose for which they are intended. Routine monitoring will require a different selection compared to a situation, of emergency.

Specimens making up the sample must be representative of the examined population. In a facility with health problem, the sample must include (a) apparently healthy fish (b) moribund fish (c) fish recently dead [30-60 minute-post mortem.] Where water currents are swift enough to cause segregation, the dead fish will be carried to the out flow. Healthy fish will be at the water intake and moribund fish are seen in outlet and intermediate position and may swim on the surface. In a stagnant pool the recently dead fish will be at bottom and to take it long handled scoop nets are necessary. Fish showing overt signs of disease are given the top sampling priority.

Sampling by lots is recommended. 'A lot is defined as fish of same age that have shared the same water supply and that have originated from a discrete spawning population.' If the population is distributed in several ponds, the sample should be composed of specimen from each pond, their proportion in the sample reflecting the relative abundance of the fish in the different ponds.

Frequency of sampling

Routine monitoring of production fish must be carried out twice a year at least, those of brood stock once a year.

Sample size

The sample size is set according to the size of population sampled.

Population size	Disease prevalence at 5%	Disease prevalence at 10%
50	29	20
100	43	23
250	49	25
500	54	26
1000	55	27
2500	36	27
5000	57	27
10,000	57	27
100,000	57	30
Over100, 000	60	30

Field sampling procedures

The nature of field sampling is determined by the situation in the facility and by available equipment. A good diagnostician can deduce the possible cause of mortality from field observations and history. The environmental stress and toxicity is characterized by non-selective mortality of fish of different age group and different species. The infections are characterized by macroscopic external / internal lesions such

as hemorrhages, swelling, ulcers, discolourations and presence of unusual fluids /secretions. Usually only one species is predominantly affected. Metazoan infections can be easily visualized.

When microbial infections are suspected it is necessary to carry out laboratory examination. It is difficult to carry out this examination at the field. There is possibility of contamination with air borne bacteria. However wet mount preparations can be examined for transmitted light, phase contrast and dark field microscopy by the necropsy examiner. Smears stained by the Grams, methylene blue, Giemsa, Wright, and /or acid-fast methods also give valuable information. Smears of necropsy material subjected to indirect immunofluorescence, ELISA, immuno-dot etc. some time gives confirmatory diagnosis.

Diagnosis of viral infections needs laboratory examination. In vitro isolation techniques are well developed for the pathogenic bacteria and many fish viruses. In suspected viral infections and bacterial infections, it is always better to send live animals to laboratories for isolation of causative agent. If live samples cannot be sent, freshly killed fish may be sent in ice. Frozen samples are not suitable for bacterial studies.

Preservation and transportation of samples

Whenever a disease out break is suspected it is advisable to send samples to specialized laboratory. Type of sample selected depends on the test to be conducted. Live samples are required for microbial/ viral studies. When moribund fishes are transported special care should be taken to keep them alive. One simple precaution is to keep the low density in containers. Fry and fingerlings can be transported in water filled polyethylene bags containing 3-5 times more air than water. The number of fish per litre of water depends on their specific oxygen requirements. A safety margin should be observed (about 25% density reduction).

When live fish cannot be supplied to laboratory, tissue samples must be supplied after proper fixation for histopathological studies. Generally gill, kidney, spleen, liver, pyloric caeca and visibly affected tissues are collected from live fish and fixed in 10% buffered formalin. Whole fish fixed is a better option than sending individual tissues.

Since putrefaction and autolysis are very fast in aquatic animals, the dead samples are not suitable for histopathological examinations. As far as possible affected fish still alive must be selected. If one cannot get live fish, only then it is advisable to take freshly dead fish. It is important to fix the tissues for histology as quickly as possible. Fixation prevents the autolytic and putrefactive change; and preserves the tissues. It also makes the tissues suitable for further procedures. Fixation is an area where people make mistakes. Poorly fixed tissue does not reveal any information and often defeats the purpose. The fixative has to reach all portion of tissue with in shortest time possible, and starts acting on the components of the tissue. So the fixative shall have high penetrating action, able to stop autolysis, putrefaction and preserve all components of tissue, as it existed in living organism. Fixative is expected to make certain favourable changes in the macromolecules which shall facilitate subsequent treatment of tissues for the demonstration of these components by staining procedures and histo-chemical reactions, but these changes shall not alter the over all structure of tissues beyond recognition. Hence, the selection of fixative is very important.

It is better to fix whole animal for histological studies. When it is not possible to send whole animal fixed vital tissues are send. Small fish [fry and fingerlings] can be fixed in 10% formalin. Small pricks are made on the body at several points before putting it into fixative. This will facilitate penetration of fixative. When larger specimens are fixed, the fish is killed by pithing or by severing the head. The viscera is opened, peritoneum above kidney removed and cuts are made on all organs. Several transverse parallel incisions are made on the body muscles. The specimens must be placed in 10- 20 times their volume of fixative [10% formalin or Bouins fluid]. The bottom and inner side of containers must be layered with absorbent cotton or blotting paper to facilitate uniform penetration of fixative. The fixative usually completes its action in 18 hours. The specimens are transported in fixative. Formalin is a general purpose fixative and produce less distortion and artifacts. However formalin gets oxidized to formic acid and formate precipitate may deposit in tissues. This is over come by washing the tissues over night in water. It is advisable to add 0.9-1.0 G Sodium chloride /100 ml of 10% formalin. 10% formalin is prepared by adding 1 ml formalin [37-40% formaldehyde solution] to 9ml of water. Another fixative is acetic formalin alcohol fixative. This consist of 10ml (10%) formalin 20ml alcohol (95%) 15ml of water and 5ml glacial acetic acid [freshly prepared]. When the sample is kept in fixative for four days in the fixative it can be taken out, wrapped in fixative soaked cotton, and placed in sealed plastic bags for transportation. It is also possible to remove samples of tissues exhibiting gross lesions from freshly killed specimens and make small pieces [3mm thick] suspend infixative. Frozen samples are neither suitable for histopathology nor for microbiological studies. Bouins solution gives good results with fish tissues. It consists of picric acid (75ml), formalin (25ml), and glacial acetic acid (5ml). Specimens should not be kept in the solution for more than a week. After the fixative the specimen has to be stored in 70% alcohol.

For crustacean tissues it is better to use Davidson's fixative, it consist of 33ml (95%) ethanol, 22ml formalin (37% formaldehyde), 11.5ml glacial acetic acid and 33.5ml water. The specimen has to be kept in fixative for 24- 72 hours, after this the specimen is stored in 70% ethanol. Since crustaceans have shell, the fixative has to be injected at several points especially at hepato-pancreatic region. Cuts are made on cephalothorax and abdominal segments. The details are given by Bell and Lightner (1988).

Importance of histopathological study

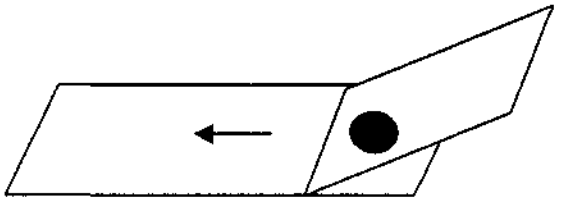
It is not always possible to precisely diagnose the disease condition by making observations or detecting presence of a microbe in association with the animal or in the environment. The main role of a disease investigator is to diagnose, treat, prevent and control the fish disease. The key to these functions is diagnosis and key to diagnosis is the ability to recognize lesions in the live dead animal, to understand their pathogenesis and through these, to make rational conclusions and recommendations for treatment, control and prevention.

Pathology is the study of the molecular, biochemical, morphological and functional aspects of disease in fluid, cells, tissues and organs of the body. The changes brought on by these aspects may leave their mark on organs, tissues and even on their mark on biochemical constituents of the body. These alterations are termed as lesion. Most of the lesions are morphological changes, which can be discerned, grossly, microscopically or ultrastructurally. The lesion is the abnormality in tissues / organs. The study of pathology involves; what is the type of lesion and how it developed. The

sequence of events from the point at which the lesion began through its entire development is called pathogenesis. It is necessary to know pathogenesis of lesion in order to make rational judgement. Pathogenesis is how the step by step progression from normal state through to the abnormal structural or functional state. The usual sequence is to find a lesion, to identify it and then attempt to determine the pathogenesis by investigation of the circumstances and sequence of events that lead to lesion. Since majority of lesions are morphological in nature, the importance of histopathological studies in disease investigation occupies the first place. Though electron microscope offers high resolution for these studies, the stringent and expensive procedures, the technical complication makes it difficult for routine studies. Another advantage of histomorphological studies is the possibility of conducting histochemical investigation and immuno- histochemical studies. The histochemical studies visualize the chemical components of tissues by which it is possible to identify biochemical lesions. These studies yield valuable information on pathology and pathogenesis. Immunohistochemical investigations use labeled antibodies to demonstrate presence specific pathogens in tissues /cells.

Blood samples

Blood samples are another material submitted to diagnostic laboratories. These should be taken as often as possible. A uniform collection method has to be followed in a farm. Cardiac puncture can be performed using 18 G or 19 G hypodermic needle and syringe. Another method is collecting from caudal artery by puncturing behind anal fin. Blood also can be collected from the dorsal aorta or by severing caudal peduncle and oozing blood is collected in a test tube. The anti coagulants used are heparin (25000 IU/Ml) and EDTA. Blood smears are immediately made after collection of blood and fixed. A drop is centered near one end of a clean slide. Another slide is placed on the median side of the drop, touching it, causing it to spread across slide. The two slides must be at an angle of approximately 45° to each other. When the blood has spread, the slide is moved along side. The blood moves with it giving a uniform thick smear. Both slides must be absolutely clean to get a good smear. The drop of blood must not be large, or the smear will be thick. The smears are air dried and fixed for 2 minutes in methanol. Giemsa, Wright or Leishman's methods stain the smears.



The other hematological test includes total erythrocyte count, total leukocyte count, erythrocyte sedimentation rate, hemoglobin percentage packed cell volume and differential leukocyte count. These estimates give a considerable information on physiological status of organism. Impression smears of cut organs like anterior kidney spleen etc. can also reveal hematological condition of fish.

Viral diseases:

Several viral diseases are reported in finfish. They include Infectious pancreatic necrosis, Viral haemorrhagic septicaemia, Infectious haematopoietic necrosis, Erythrocytic inclusion body syndrome, Erythrocytic necrosis virus infection, Lymphocystis and a number of other viral diseases.

Viruses have been associated with diseases of penaeid shrimp and crabs. Viral etiology is being attributed to nearly 20 diseases in crustaceans and 14 of these viruses have been reported from penaeid shrimps Among the viruses of penaeid shrimps, White spot syndrome, Infectious hypodermal haematopoietic necrosis, Baculovirus penaei, Monodon baculovirus, Hepatopancreatic parvo-like virus, Baculovirus midgut gland necrosis virus, Rheo-like virus, Rhabdovirus, Yellow head virus, Lymphoid organ vacuolisation virus and Taura syndrome virus are affecting the cultured shrimps. The yellow head virus reported from India appears to be a distinct virus. A number of new Bunya viruses are emerging in South East Asia. A gill-associated virus is being reported from Australia. As the aquaculture intensifies several new pathogenic viruses may arise due to mutation and spread, hence new viral infections are going to be the major threat in future.

Viruses have been implicated in mortalities of molluscs. Sindermann (1990) noted that 20 viruses have been reported from molluscan diseases. Oyster velar virus disease is a serious problem (Eaton & Wilkinson 1985). Pass *et al* (1988) found virus–like inclusion bodies in the digestive gland nuclei of *Pinctada maxima*.

Bacterial, Fungal and Protozoan diseases

Bacterial diseases have drawn considerable attention and voluminous information is available. Thirty-one bacterial species were reported to be pathogenic to fish. Among these, majority of bacteria was gram-negative belonging to species of *Vibrio, Aeromonas, Pseudomonas, Edwardsiella, Pasteurella, Flexibacter, Rickettsiales, Renibacterium* etc.

Bacteria belonging to Genus *Vibrio* are common bacterial flora associated with marine fish. *Vibrio anguillarum, V. alginolyticus, V. ordalii, V. vulnificus, V. harveyi* and *V. damsella* are pathogenic to marine fish. *Flexibacter martimus, Photobacterium damsela, Aeromonas salmonisida, Pasteurella piscicida*, and *A. hydrophila* were implicated in a number of conditions.

Rickettsias are intracellular gram negative bacteria. Rickettsia or Rickettsia-like organisms were reported from a number of fishes.

There are five species of gram-positive bacteria, which are pathogenic to fish. These include Staphylococci and Streptococci. Among the Streptococci, *S. iniae, S. shiloi, S. difficile* were found to be responsible for severe mortalities. A number of protozoan and fungal infections were reported from fishes.

In shrimps, mortalities due to bacterial infections were observed in all stages of life cycle. Most of the bacterial disease outbreaks were caused by Genus *Vibrio*. Filamentous bacterial infestations caused by *Leucothrix* were reported by a number of authors. A number of other diseases caused by rickettsia, fungi, microsporidians and epicommensals have also been reported. *V. anguillarum* and *V. alginolyticus* were reported to be causing diseases in molluscs. Rickettsia-like organisms causing disease conditions in oysters were reported by Wu and pan (2000).

Fungal infections are easily recognized. Mould like growth can be easily found out. Mycotic infections in fish are caused by fungi of genus *Saprolegina, Ichthyophonous, Achyla, Aphanomyces* etc. In shrimps *Legenidium*, and *Fusarium* are the

major fungi. Besides a number of fungi contaminate feeds with production of mycotoxins.

Prophylaxis and control of infections

Viral infections: Preventive and control measures against virus are difficult. In the case of fish viruses' vaccination and genetic selection offers some hope. However vaccine development is still under experimental stage. When epizootics occur, destruction of the entire stock and disinfections of the premises and utensils are the methods available. The introduction of virus free stock and disinfection of water supply are other methods. Strict hygiene and control of visitors are important.

Disinfection

Physical method: 1. Temperature: heat at 56° C for 2hours. 2.Radiation: UV radiation and direct sunlight kills virus.

Chemical methods: Halogen preparations like hypo chlorite, tincture iodine, 1% providone iodine etc. are powerful disinfectants. Formaldehyde and detergents are also effective. Ozone. H_2O_2 and idophore are also useful.

Control of bacterial infections: Disinfectants are effective against local infections. Antibiotics and nitro furans are given when infection cannot be controlled by other methods. This is done under expert supervision.

Mycotic infections: malachite green (0.15 ppm), Common salt and potassium permangnate (100 ppm) are the best.

Protozoan infection: The disinfection of pond before stocking. Formalin, NaCl, $KMnO_4$ calcium chloride and copper sulphate are used

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