GUIDANCE DOCUMENT FOR SETTING UP AQUATIC ANIMAL DISEASE DIAGNOSIS AND QUALITY TESTING LABORATORY

Introduction

Diseases are a major problem affecting aquaculture. Diseases may cause mass mortalities or may cause slow growth or slow mortalities over extended period. Losses amounting to several million dollars have been reported due to some diseases. Diseases may be caused by microorganisms like bacteria, viruses, fungi or parasites. Diseases caused by microorganisms are communicable diseases. Disease causing microorganisms may enter aquaculture systems through seed or live feed or water or other means. Hence following strict biosecurity measures to minimize disease is essential. We need to differentiate between "infection" which means presence of a pathogenic microorganism in an aquatic animal and "disease", which implies appearance of pathological changes or clinical signs and symptoms. In a favorable environment, pathogen, in an infected animal may remain dormant. Most often, disease is the result of the failure of the delicate balance between host, pathogen and environment. Pathogens may be of two categories: primary pathogens which may cause disease in the absence of stress or other precipitating factors and secondary pathogens, which affect an animal subjected to environmental stress (eg poor water or sediment quality) or animals infected with another primary pathogens.

Diagnosis involves identifying the nature of disease and the causative agent or factors. Thus disease diagnosis involves not only detection of pathogen but also identifying environmental factors that may lead to the disease. Environmental factors include water and sediment quality. Disease management in aquaculture involves activities related to host (eg boosting immunity), pathogen (eg biocontrol or competitive exclusion strategies) and environment (eg improving water and sediment quality to reduce stress). This guidance document is intended for those planning the setting up of disease diagnosis and aquaculture environment quality analysis.

Levels of disease diagnosis

International agencies like FAO and OIE recognize that disease diagnosis can be at principally three levels:

Level	Activity	Requirements	Personnel
I	Observation of clinical signs and symptoms in the animal at farm site, observation of pond conditions and environment. Send samples to laboratories for Level II and Level III diagnosis	Diagnostic key based on signs and symptoms, field level kits for analysis of water and sediment,	Farmers trained in observation of clinical signs of disease, field level extension staff, Persons trained in onsite diagnosis

II	Microbiological examination – microscopic observation for parasites, bacteria, fungi, histopathology, laboratory culture and identification of bacteria, fungi; antimicrobial sensitivity testing. Send samples to Laboratory for Level III diagnosis.	Basic laboratory facilities for microscopic observation of parasites, bacteria, fungi; making and staining smears, microscopic observation, laboratory cultures for bacteria, fungi; facilities for antimicrobial sensitivity testing, performing histopathology analysis, processing and application of simple diagnostic methods using formats like Lateral Flow device (LFD), storage of samples, transporting to laboratory for Level III diagnosis.	Fisheries/Aquatic animal health professionals, microbiologists, histopathologists, laboratory technicians,
III	Immunology, immunohistochemistry, molecular biology, virus isolation in cell culture.	Laboratory facilities to carry out histopathology, immunohistochemistry, real time PCR, Enzyme Linked Immunosorbent Analysis (ELISA), cell culture and virus isolation, Isothermal amplification and detection	Fisheries/Aquatic animal health professionals, virologists, molecular biologists,

Level I diagnosis involving farm site observation of clinical signs and symptoms and examination of pond conditions may be adequate to make a decision regarding the course of management. For evaluation of sediment and water quality, field level kits may be used and they could be helpful in more accurately identifying the environmental conditions that may precipitate the disease. For effectively containing the disease in a pond or farm and reducing mortalities, it is important to initiate management measures as soon as possible. Level II and Level III diagnosis may provide confirmation, but will require time. At National level, diagnostic keys for most commonly occurring diseases

should be made available to field level persons. The National Surveillance Programme for Aquatic Animal Diseases (NSPAAD) has brought out a Manual for Aquatic Animal Diseases of National Concern. This could be useful for diagnostic laboratories. Another useful document is FAO/NACA guidance document Asia Diagnostic Guide to Aquatic Animal Diseases (http://www.fao.org/3/y1679e/y1679e.pdf). The Network of Aquaculture Centers of Asia Pacific (NACA) has disease cards for various diseases of importance in Asia Pacific Region. These disease cards provide information on keys for diagnosis of these diseases. The OIE Manual of Diagnostic Tests for Aquatic Animals (https://www.oie.int/en/standard-setting/aquatic-manual/access-online/) also has a description of clinical signs and symptoms and environmental factors affecting the disease. The limitation of Level I diagnosis is that clinical signs and symptoms may be non-specific (eg lack of feeding, inactivity) and diagnosis cannot be reached in farms with animals at pre-clinical stage of the disease.

Level II diagnosis would be required to identify causative agents of disease and in the case of bacterial diseases, determining and antimicrobial susceptibility of the organism involved. Due to the emergence and spread of bacteria resistant to most commercially available antibiotics, it is very important to minimize the use of antimicrobial agents in animal production. Responsible use of antimicrobial agents in aquaculture requires accurate diagnosis and data on antimicrobial susceptibility obtained from Level II diagnostic facility. The FAO Fisheries and Aquaculture Circular 1191 (http://www.fao.org/documents/card/en/c/ca6028en) entitled "The performance of antimicrobial susceptibility testing programmes relevant to aquaculture and aquaculture products" would be a useful guide for performing antimicrobial sensitivity testing following international best practices. Histopathology involves making thin sections of the affected tissue and observing after staining to see pathological changes that are characteristic of certain pathogens such a viruses. But Level II diagnosis requires time, generally 2-3 days after sample collection. This may be too late to decide on management measures and significant losses might have occurred already before results are known. Hence, it is important to start management measures immediately after Level I diagnosis in the field side. These may be reviewed and revised after level II diagnosis. Technical expertise required to perform Level II diagnosis is much higher than that required to perform Level I diagnosis. Fisheries graduates or Aquatic Animal Health Professionals and laboratory technicians are required for Level II diagnosis. Recently, there is lot of effort to develop rapid test kits to detect pathogens without the involvement of expensive equipment's eg Lateral Flow Device (LFD) and such rapid diagnostic kits can be used in in Level II diagnostic laboratory. There are also molecular tests like isothermal amplification (eg Loop mediated isothermal amplification, commonly called LAMP) and visual detection of products, which can be performed in simple laboratories without full molecular biology facilities.

Level III diagnosis involves molecular techniques like Polymerase Chain Reaction (PCR) and various modifications such as Reverse Transcriptase PCR, Real Time PCR are commonly used. Many of the fish viruses can be grown in cell lines and virology lab is required for isolating and characterizing fish viruses. Level III diagnosis is very much essential to confirm diagnosis of viral diseases. Performing Level III diagnosis

requires fairly sophisticated laboratory and highly skilled laboratory professionals trained in molecular biology, pathology, immunology, cell culture and virology. Setting up Level III diagnostic laboratory could be expensive and running the laboratory requires high level of technical expertise.

Setting up disease diagnosis and quality testing Laboratory

Pradhan Mantri Matsya Sampada Yojana (PMMSY) support for establishment of disease diagnosis and quality testing laboratory is envisaged under beneficiary oriented activities under Centrally Sponsored component. The estimated unit cost is Rs 25 lakhs. This can be enhanced to Rs 40 lakhs if the facility includes Level III diagnosis facilities like RT-PCR and ELISA. This would enable private sector beneficiary to create facilities that can perform Level I and Level II/III diagnosis. The requirements of the laboratory may vary based on the types of analysis to be performed and these could vary depending on the type of aquaculture system for which service is intended.

For example in areas where fresh water finfish aquaculture such as polyculture of carps is to be provided with diagnostic service, the type of analysis to be performed could include:

Pond water and sediment quality parameters

pH
Water colour, turbidity
Dissolved oxygen level, salinity, alkalinity
Levels of ammonia, nitrate, nitrite, phosphates
Levels of sulphides
Predominant types of phyto and zooplankton

Pond side observation of fish

Feeding and swimming behavior,
Fish surface, gills, fins, eyes, redness, inflammation
Presence of any legions, external parasites

Laboratory examination

Microscopic examination of swabs from surface, gills for any parasites, pathogens
Examination of body cavity, organs
Examination of stained smears from suspected lesions
Bacteriological Culture of swabs from suspected lesions
Identification of bacterial isolates from suspected lesions
Antimicrobial susceptibility testing
Histopathology
Polymerase Chain Reaction analysis
Feed quality testing

Data generated by NSPAAD Phase I shows that in carp polyculture, major problems are due to parasitic and bacterial diseases. There are very few viral diseaseseg carp edema virus affecting koi carp. There are some fresh water aquaculture areas eg where

tilapia is cultured, there may be need to diagnose disease caused by Tilapia Lake Virus. There may be need for the diagnostic laboratory to either do histopathology or PCR test for viruses or arrange to send samples to Reference Laboratory for diagnosis.

In areas where shrimp aquaculture to be provided with diagnostic service, the type of analysis to be performed could include:

Pond water and sediment quality parameters
pH
Water colour, turbidity
Dissolved oxygen level, salinity, alkalinity
Levels of ammonia, nitrate, nitrite, phosphates
Levels of sulphides
Predominant types of phyto and zooplankton

Pondside observation of shrimp
Feeding and swimming behavior, examination of check tray for left over feed
Shrimp shell, appendages, hepatopancreas, gut (empty or full), any signs of inflammation, redness
Presence of any legions, external parasites

Laboratory examination
Microscopic examination of swabs from suspected lesions,
Examination of body cavity, organs
Examination of stained smears from suspected lesions
Bacteriological Culture of swabs from suspected lesions
Identification of bacterial isolates from suspected lesions
Antimicrobial susceptibility testing
Histopathology
Polymerase chain reaction
Feed quality testing

In shrimp aquaculture, viral diseases are more common than bacterial diseases and therefore in areas serving shrimp, it is important that the laboratory has capability to diagnose viral diseases. PCR kits are available for most common shrimp viral diseases and laboratory. The laboratory could either have a molecular biology section or have ability to send samples to a reference laboratory for molecular biology work.

Laboratory organization and Biosafety guidelines
Since the laboratory has to handle biological samples possibly containing pathogenic microorganisms, biosafety guidelines need to be followed in choosing location and layout of the laboratory. It is possible that laboratory is created by refurbishing an existing building or construction of new building. In both cases, it needs to ensured that the building is located in an area with road access, with assured water and power supply. Since the laboratory may use hazardous chemicals and biological material, the laboratory should be adequately separated from other building areas. For either

refurbishing an existing building or constructing a new building, advice from an architectural expert in laboratory design will be very useful in order to ensure compliance with all local building regulations and safety requirements. The laboratory should be adequately equipped with essential services and utilities, good ventilation adequate lighting, safety systems such as fire control measures, secure and protected storage for records, including computer back-ups and water and gas supplies.

PMMSY has mandated that the laboratory should have a minimum space of 1000sq ft. There should be facilities for storage of biological material and handling biohazard waste.Ideally, the laboratory should have

- Sample receiving and storage area
- Sample handling and processing area where microscopic examination eg for parasites can be done
- Microbiology section with media preparation room; inoculation and culture handling area with biosafety cabinet of appropriate safety level (eg BSL2)
- Molecular biology section
- Water, sediment and feed quality testing Section
- Waste handling area
- Office and records room

There should be adequate separation of sample receiving area, sample processing and handling area, area for performing testing chemical parameters, biosafety area for culturing microorganisms and performing antimicrobial sensitivity testing. Cross contamination between these areas should be avoided. Rest rooms and food consuming area should be away from sample receiving handling and processing area and biohazard containing area.Biological waste and chemical waste should be segregated and disposed as per national and state regulations. A common procedure followed is to have contract with an authorized waste treatment facility to collect and dispose the waste as per prevailing regulations.

Minimum space and indicative requirement for different sections of the laboratory are given below:

Laboratory Area/Section	Minimum space requirement	Indicative facilities
Sample receipt Farmer interaction	100 sqft	Access control for authorized persons Office furniture, computer, printer, document storage cabinets, hand washing facility, fire extinguisher, waste disposal containers separate for biological and chemical waste.
Sample storage	50 sqft	Refrigerator, deep freezer, storage cupboards, Fire extinguisher.

Sample handling, processing, microscopic observation Histopathology	100 sqft	Clean workbench, binocular microscope, centrifuge, refrigerator, and furniture for working staff, slide cabinets. Tissue fixation, sectioning devices
Water, sediment and feed quality testing section	100 sqft	Spectrophotometer, Water analysis system, Kjeldahl system, Titration systems, hot air oven, analytical balance, soxhlet extractor, Fume hood for handling solvents, Muffle furnace, BOD incubator
Microbiology Section		
Media preparation room	50 sq feet	Autoclave, oven, clean bench, water bath, refrigerator
Inoculation room	100 sqft	Biosafety cabinet
Incubation room	50 sqft	Incubator
Observation room	50 sqft	Clean work bench, microscope
Culture Storage room	50 sqft	Refrigerator, Deep freezer
Decontamination room	50 sqft	Autoclave, washing facility, Hot air oven
Molecular Biology room	100 sqft	Microcentrifuge, Ice flaker, PCR/Real Time PCR machine, clean work bench to handle nucleic acids, micropipettes, vortex mixer, water bath, nanodrop spectrophotometer
Office space	100 sqft	Office furniture, cupboard, computer, fire extinguishers
Staff rest room	100 sqft	Furniture, hand washing facility, toilets

An example of laboratory layout is indicated in Annex-1. But this is only an indicative design. Depending on land availability and building design, the management may consult an architect andlaboratory design consultant to alter the design without compromising the space requirements for each section.

Minimum Lab staff requirement

The Staff requirement depends on the scope of the laboratory. For most diagnostic purposes, the lab should have minimum 2 technical staff. However, higher level of lab with PCR, ELISA and unit cost of Rs 40 lakhs will need more staff as below:

Technical Lead - 1
Analyst – Microbiology and molecular biology -1
Analyst – Pathology - 1
Analyst – Water, sediment and feed chemistry - 1
Office Assistant - 1
Cleaner - 1

The analytical staff should have minimum Bachelors degree in Fisheries with training and two year experience in the concerned area – Microbiology and molecular biology or pathology or water, sediment and feed chemistry. Those with Masters degree should have undergone additional training in laboratory analysis in the concerned area. The staffs need training in quality control system as laid down in ISO 17025 General requirements for the competence of testing and calibration laboratories – resource requirements, process requirements, management system requirements. They should have attended the trainings conducted by National Accreditation Board for the Testing and Calibration Laboratories. They should have experience in preparation and implementation of Standard operating procedures, documentation, sample traceability, biosafety as well as analytical aspects, doing root cause analysis in case of failures, performing proficiency testing, participation in ring tests, calculation of uncertainties and other technical aspects like preparing for internal and external audits.

The Technical Lead should be graduate/postgraduate in fisheries with training in microbiology/pathology/molecular biology have skills in laboratory management, procurement of supplies for the laboratory, solving technical as well as personnel problems coming up in the laboratory. In addition, the Technical Lead should have five years experience in the field as analyst and training in ISO 17025 implementation, operational aspects like identifying suppliers of standards, proficiency testing materials, performing internal audit and preparing the laboratory for external audit. The Technical Lead should be able to communicate with farmers and aquaculture technical staff regarding aquatic animal health issues and advising possible management solutions.

The Office Assistant must be a graduate with skills in house management, laboratory information management system and communication with farmers and other clients.

Methodology for performing analysis

ISO 17025 allows use of internationally or nationally recognized methods that have been validated. While choosing a method, the Limit of Detection (LOD), Limit of Quantification (LOQ), sensitivity, specificity required should be a criterion. Often, there are ISO methods for most of the parameters. For chemical and microbiology analysis, use of reference materials and quality control strains would be important. For antimicrobial susceptibility testing, methodology of Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial susceptibility testing (EUCAST) should be used. OIE Manual of Diagnostic Tests for Aquatic Animals and NSPAAD Manual for Aquatic Animal Diseases of National Concern would provide internationally and nationally recognized methods.

Data reporting and interpretation

Suggested template for reporting is indicated in Annexure II. This is a general template and different laboratories may vary in the scope of analysis. The template may be changed by the laboratory according to the scope.The interpretation of results should be based on knowledge of local ecological conditions, good aquaculture practice guidelines which varies with aquaculture species and aquaculture systems and in line with the Codex Code of Practice to minimize antimicrobial resistance (CXC 61–2005).

General guidelines for good aquaculture practices are contained in the Codex Code of Practice for Fish and Fishery Products (CXC 52-2003 updated 2019) Section 6 Aquaculture Production. Tolerance of different species of fish/shrimp to water quality parameters like ammonia, sulphides, nitrite etc varies and the interpretation of these parameters should be based on the species in the system. Recommendations for management should be based on the consideration that most problems can be addressed without use of chemicals or antimicrobials by improving pond conditions. There is a need to emphasise that there is no treatment for viral diseases and recommendation could be to improve survival by improving pond conditions and taking measures to prevent spread to neighboring ponds or farms. Any use of antimicrobials should be in line with OIE Principles for prudent and responsible use of antimicrobials in aquatic animals.

Quality management in the diagnostic and quality testing laboratory

If the laboratory is to deliver valid test results, it is important to follow quality management system in accordance with principles enunciated in OIE Manual of Diagnostic tests for Aquatic Diseases. The main elements are:

- Clear description of the scope, responsibilities and goals of the laboratory
- Try to meet managerial and technical requirements laid down in ISO/IEC 17025 standard on the General Requirements for the competence of testing and calibration laboratories.
- Determine the scope of the quality management system and/or of the laboratory accreditation.
- All equipments should be regularly calibrated according a pre-determined schedule.
- Try to implement quality assurance, quality control protocols according to ISO/IEC17025 using certified reference materials as standards and participate in proficiency testing.
- Adopt internationally validated test methods and implement measurement of uncertainty and estimate uncertainty for each method.
- Adopt measures for continued implementation of quality management according to a strategic planning.

As far as possible, the laboratory should adopt test methods recommended by OIE Diagnostic Manual orNSPAAD Manual. For diseases that are not OIE listed, test methods recommended or validated by other international or national agencies could be used.

Link to National Reference Laboratories and Aquatic Referral Laboratories

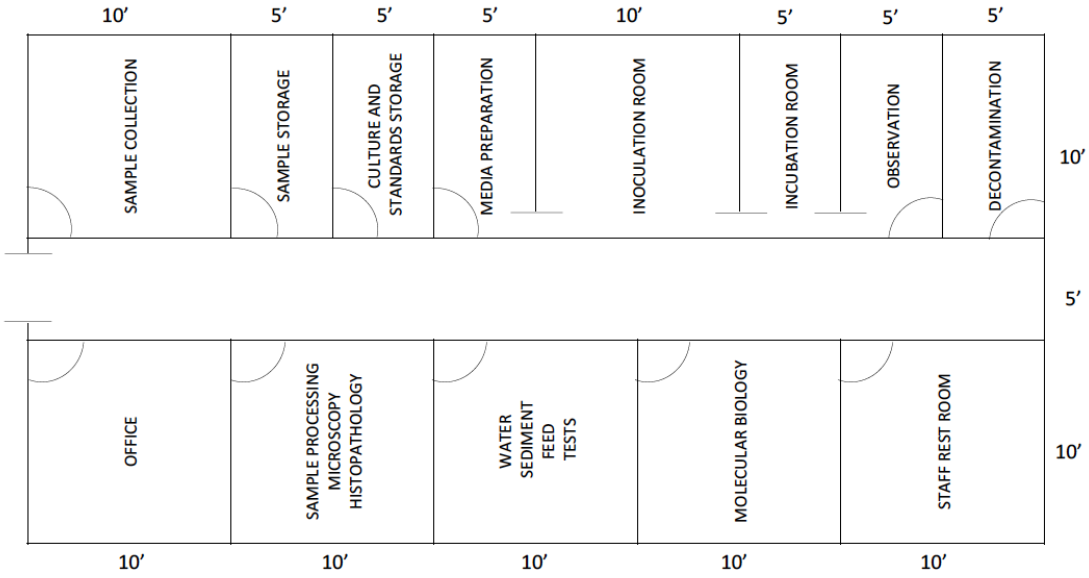
Presently, the National Bureau of Fish Genetic Resources (NBFGR), Lucknow is the nodal institute for NSPAAD and is the nodal center for supply of reference material and training in aquatic animal disease diagnosis. There are other ICAR Institutes with specific capabilities eg Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar with high level of expertise in diseases of freshwater fish and Central

Institute of Brackish water Aquaculture (CIBA) has expertise in diseases of shrimp and brackish water fish. Aquatic disease diagnostic laboratories can refer to these laboratories for help in arriving at diagnosis and improve the expertise of the staff. The diagnostic laboratories can also utilize expertise available at ICAR institutes and Colleges of Fisheries, which are part of NSPAAD network.Aquatic Referral Laboratories being established under PMMSY would be an important reference source. Further, there are OIE reference Centers for different diseases that my be source of reference material and could be consulted.

Suggested checklist for disease diagnosis and quality test laboratory

Annexure III provides a checklist for basic minimum infrastructure and staff facilities required to establish a laboratory. This checklist can also be used for evaluating proposals for establishing the laboratory.

Annexure 1
Suggested layout for the laboratory and space requirements for different sections



In this design, there is requirement of building with a length of 50 ft and breadth of 25ft. In this design, the entry to each section is separate through the passage. Entry to microbiology section is to media preparation room and exit from observation room. The decontamination room is at the end of the building and so also is the staff rest room. After consultation with an architect and laboratory designer, the layout can be changed without sacrificing the space required for each room.

Annexure II

TEMPLATE FOR REPORT FROM DISEASE DIAGNOSIS AND TESTING LABORATORY

Sample and analysis	Details/Result	Comments
Sample: Sample submitted by: Date and time of sample collection: Date of and time of receipt of sample in the Laboratory: Sample received by: Sample ID: Date of analysis: Date of reporting:		
Clinical history Field level observations Pond conditions Clinical signs and symptoms		
Analysis performed Eg**For Water** Turbidity Dissolved oxygen level pH Total dissolved solids Total suspended solids Ammonia level Nitrate level Nitrite level Alkalinity Phosphates Sulphide level Total plankton count Phytoplankton Zooplankton Eg**for Sediment** pH Sulphide level Ammonia level Total organic carbon Eg**for Feed** Proximate composition Aflatoxin		

Other parameters Eg **Microscopic examination of animal tissue** Presence of parasites Presence of fungal Hyphae Gram stain		
Histopathological observations Section of: hepatopancreas/gut/gills etc Observations Possible conclusions		
Microbiological analysis Tissue: Microscopic observation, gram stain: Bacterial culture/Fungal culture Colony morphology Gram stain Biochemical characters Identification Antimicrobial sensitivity testing Conclusions		
Molecular Biology analysis Test performed: PCR/ Real Time PCR for --- Observations: Conclusions		
Overall conclusions		

Signature of the Analyst

Disease diagnosis and management recommendations:

Signature of the Technical Manager

Annexure III

CHECKLIST FOR THOSE SUBMITTING PROPOSALS FOR SETTING UP AQUATIC ANIMAL DISEASE DIAGNOSIS AND QUALITY TESTING LABORATORY

Item	Status	Comments
Location of the laboratory with road, power and water access		
Location of the laboratory appropriate for biosafety implementation		
Laboratory space availability: • Sample receiving and storing area • Sample handing and microscopic examination area • Pond water, sediment and feed analysis section • Microbiology Section with biosafety cabinet • Histopathology section • Molecular biology section • Biohazard and chemical hazard storage and decontamination • Office space and records keeping area • Staff room and rest rooms		

Basic laboratory equipments
• Electronic balance
• Binocular microscope
• Centrifuge
• Spectrophotometer
• Kjeldahl system
• Soxhlet extractor
• Titration system
• Biosafety cabinet
• Clean bench for molecular biology
• Fume hood
• Autoclave
• Incubator
• Clean bench for media preparation
• Hot air oven
• Histopathology equipment
  ○ Sample fixation
  ○ Sectioning
  ○ staining
• Water and sediment quality testing device (field kits)		

• Spectrophotometer
• Molecular biology section
  ○ Real time PCR machine
  ○ Centrifuge
• Refrigerator
• Deep freezer		

Personnel
• Technical Lab Manager
• Qualified fisheries graduates with experience as analysts
  ○ Histopathology
  ○ Microbiology and molecular biology
  ○ Water, sediment feed anaysis
• Office assistant		

Other facilities available		
Training in biosafety		
Training undergone in disease diagnosis and pond water, sediment and feed quality testing		
Lab manual and Standard Operating Procedures		
Training in quality management as per ISO/ICE 17025		
Linkage with ICAR institute/College of Fisheries		