

6 DISEASE DIAGNOSIS

6.1 Purpose

The material presented in this section supports Section 6 of the *Technical Guidelines*. Diagnosis requires several levels of data, starting with farm- or site-level observations and progressing in technical complexity to electron microscopy, immunology, nucleic-acid assays and other biomolecular methods. This means that all levels of expertise, including that of the farmer and extension officer, make contributions that are critical for rapid and accurate disease diagnosis. The *Technical Guidelines* deliberately emphasize capacity building (facilities and expertise) for basic diagnosis and surveillance at the farm level (Level I). This is the essential foundation for early disease detection and implementation of effective response protocols that can minimize social and economic losses.

In addition to disease diagnostic input levels, this section further describes the diagnostic issues that relate specifically to the movement of live aquatic animals.

Regional and national policy makers need to consider disease diagnosis at all of the three levels agreed-upon by the countries participating in the development of the *Technical Guidelines* (see Section 6 of the *Technical Guidelines* and Table 6.1, below). Countries can move from one level to the next as they build up their infrastructure, capacity and experience.

6.2 Levels of Disease Diagnosis

The accurate diagnosis of aquatic animal diseases requires different levels of disease surveillance and data collection, ranging from farm-site observations through to the use of state-of-the-art diagnostic technology. Development of expertise at each level of diagnosis requires investment in training and infrastructure, with successive levels requiring more complex training and greater financial resources. Table 6.1 shows the investment required at the three different levels of disease surveillance (termed Levels I-III). Some countries will need to increase investment to meet diagnostic requirements for listed diseases which need Level II and III capability for their identification and/or confirmation. Where such diseases (or the potential for their introduction) have limited probability of occurrence, diagnostic/surveillance assurances can be achieved by enhanced links with the required diagnostic capabilities in other participating countries. For OIE-listed diseases (see Annex V), OIE Reference Laboratories can be used. For other diseases of regional concern (see additional diseases listed in Annex VI), Regional Resource Centers (RRCs) can be consulted (see Section 6.4). It is important to note, however, that all three levels of diagnostics capability are necessary for the diagnosis of new or rare diseases.

As shown in Table 6.1, Level I activities include farm-site monitoring and provide information essential for making presumptive diagnoses. In some cases, this may provide sufficient information to make adequate health management decisions (see Box 6.1). Level I diagnostics is especially valuable for compiling complete case-histories which can accompany and assist diagnosis of samples submitted for Level II or III diagnosis (see Box 6.2). Such information helps focus diagnostic effort, enhancing speed and accuracy of results. Level I diagnosis is generally appropriate for:

- macroscopic ectoparasites, which are easily identified;
- diseases with specific gross pathology; and
- farms/sites with an established history and/or susceptibility to specific diseases.

Although Level I diagnostics rarely results in a conclusive disease diagnosis, it provides an essential starting point for reducing the risk of pathogen transfer via movement of live aquatic animals. Microbial or internal infections are generally not readily detectable using Level I techniques. Furthermore, latent (sub-clinical) infections may, in some cases, also evade diagnosis at Level II and III. However, Level I monitoring activities provide information

Box 6.1. Examples of diseases that can be diagnosed using Level I capability.

Black Splint Syndrome of *Penaeus monodon* (and possibly other penaeids), caused by *Vibrio vulnificus*, results in pathognomic (infection-specific) gross clinical signs. These consist of blackening along the sub-cuticular connective tissues. This makes confirmatory diagnosis reliable at Level I, directs attention towards pond management, rather than an infectious disease and can be used to permit movements.

Agmasoma sp. in penaeids causes "cotton shrimp," which is characteristic of microsporidian diseases. Diagnosis is possible using gross observation of muscle whitening (Level I). This is sufficient to initiate remedial husbandry, such as removal of fish from the culture system.

essential for detecting emerging clinical infections (e.g., deviations from the established "normal") thus, they should not be undervalued in any diagnostic program. In some cases, they also provide information vital to meaningful interpretation of laboratory findings derived from Level II and III activities. Conscientious observations and recordings of shipments or receipt of grossly diseased aquatic animals is also frequently sufficient to provide a presumptive diagnosis which will lead to prevention of disease spread or transfer. Thus, all countries should ensure that Level I diagnostic capabilities are well-established, in addition to obtaining access to and/or developing Level II and III resources (within or outside the region/country).

Level II diagnostics is required for diseases whose clinical signs could be caused by a variety of infectious (and non-infectious) agents. Level II (and occasionally, Level III capability) is also required for external and internal pathogens that are not readily recognized by gross examination using the naked eye (e.g., microbial agents, many types of parasites). In these cases, bacteriology (culture characteristics, chemical profiles or light microscope examination), mycology (as for bacteriology) or histology (preserved and stained sections of tissue 5 micrometers thick for microscopic examination of cell structures) (see Box 6.2 - EUS example) is required. In addition, many parasitic infections are impossible to identify accurately without specialized morphological study (e.g., trichodinid ciliates; most monogeneans, digeneans and cestodes). Bacteriological, mycological and parasitological investigations all pivot on association with disease signs. In some cases, however, the cause of clinical disease signs cannot be identified from lesion smears, cultures or squashes (see Box 6.3). In these cases, tissue samples have to be collected and preserved for light (or Level III electron) microscopy. As with bacteriology, mycology and parasitology, trained expertise and equipment is necessary. This is particularly true for many molluscan and crustacean diseases, where tissues may have to be collected for virology and/or electron microscopy (Level III activities). Personnel involved in Level II diagnostics require specialized training and access to necessary equipment.

Where personnel have not had dedicated undergraduate training in such diagnostic techniques, the period required to gain independent capability and diagnostic confidence can exceed two years. This includes a

period where diagnosticians have to establish "normal" base-line references and material. With undergraduate technical training, the period is shortened (6 months - 1 year workplace training), since the personnel only require slight adaptation of techniques and familiarization with aquatic animal pathogens. Introductory workshops for personnel with some aquatic animal health background can further shorten Level II training. As with other Levels, all Level II training requires linkage to specialists and established reference resources. This is most easily achieved via the Internet; however, additional provisions must

Box 6.2. Presumptive vs. confirmatory diagnoses – example: Epizootic Ulcerative Syndrome.

EUS causes grossly visible skin lesions. These first manifest as small red spots, which progress to acute dermatitis. The raised, whitened edges of such lesions can be used, with Level I observation, to diagnose *presumptive* EUS in species such as snakehead.

The presence of the oomycete fungus *Aphanomyces invadans* is recognized as the essential component for confirmatory diagnosis of EUS. This requires histopathological preparations to detect and identify the characteristic fungal hyphae of *A. invadans* along with associated granulomatous lesions and surrounding epithelioid cells.

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be made for trainees/employees who do not have access to this communication infrastructure.

Level III diagnostic capabilities are required for problematic pathogens and those that are difficult or impossible to identify at Levels I and II. As noted above, this is especially applicable to sub-clinical infections. Level III training requires more experience than Level II. Electron microscopy requires acute attention to preparation details, as well as awareness of normal sub-cellular structures in different tissues from different hosts. Immunological and molecular diagnostic techniques require a refined background knowledge of normal host and pathogen physiology and genetics, as well as extreme sensitivity to contamination which can affect results. This is particularly important where whole infectious organisms or *in situ* evaluation of pathology is not a component of the diagnosis. Classic virology requires knowledge of the differing maintenance requirements of living cell-lines for intracellular pathogen isolation and culture, as well as detailed knowledge of virogenesis, cytopathological effects (CPE) and molecular virology. These diagnostic fields have only developed relatively recently for aquatic organisms, and classic techniques are lacking for invertebrate hosts, thus, training in this area is particularly extensive and specialized.

Development of competency at each level of diagnosis is the basis for effective export certification. In many countries, such certification is currently based on Level I diagnosis (e.g., visual examination and country/enterprise history of disease), or no aquatic animal health training. Development of diagnostic competence will allow more accurate pre-export surveillance and diagnosis, and will result in a significant improvement in the assurances currently provided by many certification systems.

Box 6.3. Evolution of diagnostic confidence with level of diagnosis – white spot syndrome virus (WSSV).

Diagnosis by gross observation of white spot lesions was initially considered sufficient, until other etiological agents were discovered to cause similar lesions. This reduced confidence for first time diagnoses.

Subsequent Level II diagnosis achieved through histology and microscopic observation of intranuclear inclusion of herpesvirus bodies in gill epithelial cells as well as elsewhere (see ADG). This is sufficient to confirm diagnoses done at Level I. Both levels are insufficient, however, for sub-clinical carriers of the virus.

Level III diagnosis using DNA probes for various molecular diagnostic techniques provides the ultimate diagnostic confidence (to date) for positive and negative cases.

6.3 OIE Reference Laboratories

Diagnostic capability and specialized expertise on specific diseases and disease agents is best developed at laboratories with day-to-day experience with these diseases (usually laboratories in enzootic areas for each disease). In recognition of this, the OIE has designated laboratories with such expertise in OIE-listed diseases as “OIE Reference Laboratories.” These are listed on the OIE website (http://www.oie.int/diseases/A_list.htm). With website access, a diagnostician can click on a disease of concern and find the OIE-approved reference laboratory contact information. Since some laboratories are located in areas with more than one OIE-listed disease, they may function as Reference Laboratories for each of these diseases.

The role of an OIE Reference Laboratory is:

- to co-ordinate/conduct surveillance for the specific listed diseases they are responsible for;
- to provide diagnostic confirmations for material submitted by other laboratories which are believed to have suspect or presumptive infections; and
- to ensure that diagnostic methodologies for the specific disease agents are regularly evaluated and improved, as required, through appropriate research.

These responsibilities are undertaken with government support from the country, union, or region with such reference laboratories. OIE may provide supplementary support, however,

reference capability is considered to be provided on stand-alone resources. Laboratories wishing to be considered as OIE Reference Laboratories submit applications to OIE; OIE cannot request or demand Reference Laboratory services.

6.4 Regional Resource Centers

Many non-OIE-listed diseases are of regional concern, with respect to accurate diagnosis, as well as trans-boundary trade. As with the OIE-listed diseases, laboratories with strong capabilities and established expertise with such diseases are those which have to deal with them on a regular basis. This means that equally competent laboratories in areas where the disease does not normally occur, or those laboratories in the process of developing such capability, may lack the requisite experience for diagnostic confidence for these diseases. In such instances, Regional Resource Centers (RRCs) should be made available for sample submission by laboratories/field sites making presumptive diagnoses (Level I or II), as well as to provide "second opinion" confirmations for Level II/III laboratories that have diagnosed the disease/disease agent in question. Such RRCs would participate in this program voluntarily through application for inclusion on a laboratory referral list maintained by NACA Headquarters. They could function, additionally, as RRCs for training in diagnostics (all Levels) for the disease(s) for which they have expertise.

Basic criteria for recognition as a Regional Resource Center are:

- At least five years experience in diagnosing and studying the disease(s)/pathogen(s) for which the RRC application is made.
- Presence of more than one diagnostician (scientist, biologist or technician) with competence in the disease(s) in question. Where such is not the case, the laboratory can be listed, but should that specialist leave the laboratory, it must withdraw its RRC services.
- Ability to accept without charge samples submitted for diagnostic confirmation of infection by the pathogen(s) for which RRC designation has been given.
- Ability to provide confirmatory diagnosis (or re-directed diagnosis, as appropriate) to the submitter (laboratory, farm site, government authority) within 3-4 weeks (or in the shortest period of time required to apply confirmatory diagnostic techniques).
- Easy accessibility by standard rapid communications avenues (telephone; fax; e-mail).
- Willingness to host training workshops on a regular basis (annually or bi-annually) in diagnosis (at all Levels) of the disease(s)/pathogen(s) for which the RRC is recognized.

6.5 Capacity and Institutional Implications

The requirements for each diagnostic Level are described in Table 6.1. At Level I, the best training is experience. Apprenticing (shadow training) of young/inexperienced personnel on farm sites with farm workers or managers, is frequently sufficient to provide the capability to distinguish significant losses from routine losses, as well as abnormal from routine mortalities. If fishery extension officers and local fishery/aquaculture biologists can be included in such "orientation" training, this will enhance collaborative efforts, as well as communication links. This applies also to aquatic veterinary support.

In order to minimize the risk of trans-boundary pathogen transfer, it is important that some level of harmonization in basic diagnostics be established within the Region. Considering the significant differences in diagnostic capacity and infrastructure, countries within the region should attempt, at least as a starting point, to develop Level I diagnostic capabilities for the diseases and disease agents on the NACA/FAO and OIE Regional Disease Reporting Lists (Annex VI), as well as basic recognition of clinical pathology associated with known serious pathogens. Such information is available through regional diagnostic manuals and the *Asia Diagnostic Guide to Aquatic Animal Diseases*.

Table 6.1. Diagnostic case-history contribution levels.

Level	Activity	Work requirements	Responsibility	Technical requirements to support activities
I	Observation of Animal and Environment Gross Clinical Examination	Knowledge of normal health status (feeding, behavior, growth) of stock. Frequent/regular observation of stock. Regular, consistent, record-keeping and maintenance of records – including fundamental environmental information. Knowledge of contacts for health diagnosis assistance (Level II, III). Ability to submit and/or preserve representative specimens for optimal diagnosis.	Farm workers/ managers Fishery extension officers On-site veterinary support Local fishery biologists Quarantine Inspectors	Field keys, farm record keeping formats, equipment lists, model clinical data sheets, pond-side checklist. Protocols for sample preservation/transport for Level II/III examinations. Model job descriptions/skill requirements. <i>Asia Diagnostic Guide to Aquatic Animal Diseases</i> . Access links to Level II and Level III.
II	Parasitology Bacteriology Mycology	Laboratories with basic equipment and personnel trained/experienced in aquatic animal pathology. Keep and maintain accurate diagnostic records. Preserve and store specimens. Knowledge of/contact with different areas of specialization within Level II. Knowledge of who to contact for Level III diagnostic assistance.	Fish biologists/ technicians Aquatic veterinarians Parasitologists/ technicians Mycologists/ technicians Bacteriologists/ technicians Histopathologists/ technicians	Model laboratory record-keeping system. Protocols for preservation/transport of samples for other Level II and Level III analysis. Model laboratory requirements/equipment/consumables lists and model job descriptions/skill requirements. Contact information for Level II and Level III expertise. Access to <i>Asia Diagnostic Guide to Aquatic Animal Diseases</i> ; <i>OIE Diagnostic Manual for Aquatic Animal Diseases</i> ; regional general diagnostics manuals. Access links to Level I and Level III resources.
III	Histopathology Virology Electron Microscopy Molecular Biology Immunology	Highly equipped laboratory with specialized and highly trained personnel. Keep and maintain accurate diagnostic records. Preserve and store specimens. Maintain contact with people responsible for sample submissions.	Virologists/ technicians Ultrastructural histopathologists/ technicians Molecular biologists/ technicians	Model laboratory requirements/equipment/consumables and model job descriptions/skill requirements. Contact information for reference laboratories. Protocols for sample preservation for consultation/validation. Access to <i>Asia Diagnostic Guide to Aquatic Animal Diseases</i> ; <i>OIE Diagnostic Manual for Aquatic Animal Diseases</i> ; and molecular and microbiology diagnostic references. Access links to Level I and Level II resources.