14. Diagnostic procedure in the case of mortality caused by unknown aetiology

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Table of contents

14.1. Introduction

European legislation on aquatic animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals sets criteria to investigate “increased mortality” (EU, 2006; EU, 2008; OIE, 2018). “Increased” means significantly above the level of what is considered to be expected for the farm under the prevailing conditions and is decided in cooperation between the farmer and the competent authority. In accordance with legislation, increased mortality must be investigated to understand the cause, rule out listed pathogens and allow early detection and definition of emerging

disease. An emerging disease is defined by OIE, in "The Aquatic Animal Health Code" (OIE, 2018) as "a disease, other than listed diseases, which has a significant impact on aquatic animal or public health resulting from a change of known pathogenic agent or its spread to a new geographic area or species; or a newly recognized or suspected pathogenic agent." A close collaboration between the diagnostic laboratory, the fish health manager and the fish farm will streamline the process and facilitate solving the issue (Patarnello and Vendramin, 2017). The steps to be considered and taken are explained in the following.

14.2. Case definition

A first important assessment is needed to define whether an infectious agent is suspected or other stressors are primarily involved in the mortality outbreak, such as oxygen failure in the tank, algal bloom around a sea cage farm, toxic leaks etc.

If an infectious aetiology is suspected, it is necessary to provide a specific case definition in order to define the disease outbreak. This will include all anamnestic information related to the disease outbreak including data on the host, the environment, farming practices and preliminary identification of an aetiological agent, if available;

Host: fish species, biological stage (larval, fry, juvenile, brood stock), organ affected, clinical signs, common necropsy findings need to be listed.

Environment: time of the year, water temperature, abnormal meteorological conditions (storms, sea currents etc.) should be recorded.

Farming practices: feeding and stocking procedures, vaccination, antiparasitic treatments etc.

The preliminary characterization of the pathogen is related to the infective agent and can include observation of microscopic parasites in fresh preparation, isolation of bacteria on agar culture, viral isolation on cell culture and thorough histopathological description of the affected organs. For the investigation of specific pathogens please refer to the other chapters in this book and the OIE "Manual of Diagnostic Tests for Aquatic Animals" (OIE, 2019).

14.3. Sampling procedures

Standardized sampling procedures are of paramount importance when investigating a disease outbreak. One possible approach is to sample the same group of fish over time, but it is rarely applied due to the urgent need to implement treatment and control measures to stop the mortality. An alternative approach may be to "capture" different stages of the disease by sampling different groups on the same site.

A standardized approach for sampling, which has been proven effective, consists of collecting fish from three strata (discrete groups that compose the whole farm population):

- Sick fish in an affected production unit (for example a tank or a cage).
- Healthy fish in an affected production unit.
- Healthy fish in a non-affected unit.

When approaching a disease outbreak, it is important to sample at least 5 fish per group, the moribund fish will be selected instead of dead fish in order to avoid post-mortem alterations and proliferation of unspecific organisms in the carcass. When sampling for the investigation of emerging disease, pooling of different specimens should be avoided.

Sampling represents the first phase and a bottleneck in the diagnostic process. It has to be seriously considered, planned with the laboratory and conducted by collecting and preserving material for different analyses. For this reason, it is recommended to collect and store samples

from the same tissue/fish in different ways to allow multiple analyses enabling a precise picture of the disease situation. Ideally, all samples should be collected, fixed and preserved as follows:

- preserved in RNA for molecular analysis later,
- fixed in formalin for histopathology,
- preserved in transport MEM for viral isolation,
- collected by swab for bacteriology,
- maintained frozen for NGS, infectious trial, back up etc.

14.4. Diagnostic procedures

The aim of the diagnostic investigation, specifically in cases of unexplained increased mortality, is to establish a chain of evidence establishing solid links between the clinical signs, necropsy findings, histopathological findings, microbiological tests and specific tests leading to an explanation of the cause of the mortality. In this regard, it is important to keep an open approach and consider that the disease picture observed could be produced by multiple pathogens persisting on the same fish stock. As a general guideline, it is never appropriate to identify a new pathogen relying only on one method and initial identification of pathogens shall be corroborated with different methods.

The following instructions refer to the sampling scheme described above, which includes 5 fish from 3 different strata of a farm, including affected fish in an affected production unit (for example a tank or a cage), healthy fish in an affected production unit and healthy fish in a non-affected unit. The descriptions are based on protocols from the "Manual of Diagnostic Tests for Aquatic Animals" (OIE, 2019).

14.4.1. Histopathology

Histopathological investigation helps to understand the host response to the pathogen, in order to increase the understanding of the effect of the pathogen. Proper sampling is critical to obtain high-quality information from the investigation and avoid artefacts.

Fish larvae, tissue or organs should be fixed in 10% buffered, neutralized formalin for at least 24h.

Each sample has to be preserved in fixative 10 times the volume of the sample.

- For larval stages, with body thickness below 5 mm, the whole fish can be fixed
- For small fish, with body thickness over 5 mm, the abdominal wall and the gill operculum on one side must be removed prior to fixing the whole carcass in formalin.
- For large fish, single organs should be taken out during the necropsy and placed in a container with a fixative. Samples should be of no more than 5 mm thickness and 1-2 square centimetre surface.

The tissue samples should include the area with evident lesions, including the area of transition from normal to affected tissue. Furthermore, independently of the necropsy findings, the following organs and tissues should be sampled: gill, heart, liver, gastrointestinal tract, pancreas, spleen, kidney, an area of the flank including skin-side line-muscle.

14.4.2. Virology

Virus isolation on cell culture can allow detection of viable viral particles, which, if found, is crucial in the diagnostic work. The procedure involves the collection of material, inoculation of cell lines and possibly sub-cultivation.

14.4.2.1. Collection of diagnostic material

Before shipment or transfer to the laboratory, pieces of the organs to be examined must be removed from the fish with sterile dissection instruments and transferred to sterile plastic tubes containing transport medium, i.e. cell culture medium with 10 % calf serum and antibiotics. The combination of 200 i.u. penicillin, 200 µg streptomycin, and 200 µg/ml kanamycin can be used, but other antibiotics of proven efficacy may be used as well.

Pieces of organs should be collected in one sterile tube containing a sufficient amount of the transport medium (approximately 10 times the weight of the tissue sample). It is considered acceptable for the analysis to pool the sample from the same stratum. The tissue in each sample should weigh a minimum of 0.5 g.

The material to be used for virological examination depends on fish size:

- Fish below 4 cm (fry) can be used whole. For some viruses, it is recommended to use only the internal organs, therefore consult the OIE Diagnostic Manual (OIE, 2019) for the specific disease under suspicion.
- For fish between 4 cm and 6 cm, the internal organs may be used, after removing the head and caudal fin.
- For larger fish, the kidney, spleen and heart may be used. When a chronic disease is suspected, the brain may be included as well. Other organs can be included depending on which virus is suspected. For example, if there is a suspicion of nodavirus, only the brain should be used.

During transport, the tubes should be placed in insulated containers (for instance, thick-walled polystyrene boxes) together with sufficient ice or cooling elements to ensure chilling of the samples during transportation to the laboratory. Freezing should be avoided. The temperature of a sample during transit should never exceed 10 °C and ice should still be present in the transport box at delivery, or one or more freeze blocks must still be partly or completely frozen.

The virological examination should start as soon as possible and not later than 48 hours after the collection of the samples. Whole fish may be sent to the laboratory when the temperature requirements during transportation can be fulfilled. The fish should be wrapped in absorbent paper and shipped in a plastic bag to avoid cross-contamination. Live fish may be shipped as well. All packaging and labelling must be performed in accordance with national and international transport regulations as appropriate (see Section 2.2).

14.4.2.2. Collection of supplementary diagnostic material

Other fish tissues may be collected as well and prepared for supplementary examinations, according to the agreement with the diagnostic laboratory involved.

14.4.2.3. Freezing in exceptional cases

Where practical difficulties arise (i.e. bad weather conditions, non-working days, laboratory problems, etc.) which make it impossible to inoculate cells within 48 hours after the collection of the tissue samples, it is acceptable to freeze the tissue specimens in cell culture medium at -20 °C or, preferably, at -80 °C, and carry out virological examination within 14 days. The tissue, however, must be frozen and thawed only once before the examination. Some viruses may not sustain freezing (e.g. IPNV), so always consider what the sample will be examined for, prior to freezing the samples.

14.4.2.4. Cell lines to be included

A comprehensive panel of cell cultures should be included when investigating a new disease outbreak. Cell lines originating from the same species of fish under investigation should be used whenever available. As a minimal requirement, the panel should include Bluegill fry cell line -2 (BF-2) or Rainbow trout gonad cell line - 2 (RTG-2) and either Epithelioma papulosum cyprini (EPC) or Fathead minnow (FHM). The panel may be expanded with striped snakehead (SSN-1) cell line or similar (E-11, GF-1 etc.), and with Atlantic salmon kidney (ASK) and Chinook salmon embryo (CHSE) cell lines. Cells shall be grown at 20 to 30 °C in a suitable medium, namely Eagle's minimum essential medium (MEM) or modifications thereof, with a supplement of 10 % foetal bovine serum and antibiotics in standard concentrations. When the cells are cultivated in closed vials, the medium shall be buffered with bicarbonate. The medium used for cultivation of cells in open units may be buffered with tris (hydroxymethyl)aminomethane-HCl (Tris-HCl) (23 mM) and sodium bicarbonate (6 mM). The pH must be 7.6 ± 0.2. The cell cultures to be used for inoculation with fish tissue material shall be young, normally 1-day old cell culture monolayers where possible; however, a range between 4 to 48 hours old may be accepted. The cells must be actively growing at inoculation.

14.4.2.5. Inoculation of cell cultures

Antibiotic-treated organ suspension shall be inoculated into cell cultures in two dilutions, namely the primary dilution and, in addition, a 1:10 dilution thereof, resulting in final dilutions of tissue material in the cell culture medium of 1:100 and 1:1000, respectively, in order to prevent homologous interference. At least two cell lines shall be inoculated as referred above. The ratio between inoculum size and volume of cell culture medium shall be about 1:10. For each dilution and each cell line, a minimum of about 2 cm^2 cell area, corresponding to one well in a 24-well cell culture tray, shall be utilized. Cell culture trays shall be used where possible.

14.4.2.6. Incubation of cell cultures

The incubation temperature of cell culture varies in relation to the tolerance of cell cultures, and the optimal growth temperature of the aetiological agent. Inoculated BF-2, EPC, RTG, FHM, ASK and CHSE cell cultures shall be incubated at 15 °C. For SSN-1, E-11 and GF, the temperature of incubation should be 25 °C. Inoculated cell cultures have to be incubated for 7 to 10 days. If the colour of the cell culture medium changes from red to yellow indicating medium acidification, pH adjustment with sterile bicarbonate solution or equivalent substances must be performed to ensure cell susceptibility to virus infection. Inoculated cell cultures must be inspected regularly by microscopy, at least three times a week, for the occurrence of CPE at 40 to 150× magnification. If obvious CPE is observed, virus identification procedures should be initiated immediately. In this case, remaining cell supernatant and cell lysate shall be stored frozen at -20 °C or, preferably, at -80 °C.

14.4.2.7. Subcultivation

If no CPE develops after the primary incubation for 7 to 10 days, subcultivation must be performed on fresh cell cultures utilising a cell area similar to that of the primary culture. Aliquots of medium (supernatant) from all cultures or wells constituting the primary culture shall be pooled according to cell line 7 to 10 days after inoculation. The pools shall then be inoculated into homologous cell cultures undiluted and diluted 1:10 (resulting in final dilutions of 1:10 and 1:100, respectively, of the supernatant) as described in the point above. Alternatively, aliquots of 10 % of the medium constituting the primary culture shall be inoculated directly into a well with fresh cell culture (namely, well to well subcultivation). The inoculated cultures shall then be incubated for 7 to 10 days at 15 °C (salmonid cell lines) and 25°C for SSN-1 and inspected as described above. If toxic CPE occurs within the first 3 days of incubation, subcultivation shall be performed at that stage, but the cells shall then be incubated for 7 days and subcultivated again

with further incubation for 7 days. When toxic CPE develops after 3 days, the cells shall be passed once and incubated to achieve a total of 14 days from the primary inoculation. There must be no evidence of toxicity in the final 7 days of incubation. If bacterial contamination occurs despite treatment with antibiotics, subcultivation shall be preceded by centrifugation at 2 000 to 4 000× g for 15 to 30 minutes at 2 to 5 °C, or filtration of the supernatant through a 0.45 µm filter or both (low protein-binding membrane). In addition to this, subcultivation shall follow the same procedures as described for toxic CPE in the fourth paragraph of this point. If no CPE occurs, the test may be declared negative.

14.4.3. Bacteriology

The bacteriological examination should be carried out by inoculating on agar plates, samples taken from kidney, brain and from the area where wounds or lesions are present and consistent with the case definition. Sampling and inoculation are done with a sterile loop. The selection of agar plates should at least include blood agar (BA), marine agar (MA) and cysteine heart agar with blood (CHAB).

Agar plates should be incubated at 15 °C and 20 °C for at least 7 days, in an appropriate incubator and away from contamination sources. Each plate must be labelled with the lot, the sample origin, the species and the organ from which the sample was taken. In case of slow-growing bacteria, such as intracellular bacteria, it is appropriate to incubate the plates for longer periods.

Once the microorganism is isolated, it can be characterized and identified by:

- Microscopy and staining. These methods (including among others Gram staining, etc.) give an indication of the shape, morphological features and motility of the bacteria.
- PCR and sequencing. Generic primers for 16 S rRNA sequencing can provide initial and partial identification of newly isolated bacteria.
- MALDI-TOF enables rapid identification of pure bacterial colonies. This method requires a validated database of reference strains to correctly identify the bacteria

14.4.4. Molecular biology

The introduction of NGS (Next Generation Sequencing) and sequence-independent single primer amplification has recently led to the discovery of a number of nucleic acid sequences originating from viruses and other microorganisms which are yet to be classified. A large number of these new microorganisms will have a big impact on the taxonomic order used today, and furthermore, greater focus is required on assessing their biological relevance to farmed animals. Among other fish pathogens recently identified by NGS, are Piscine Myocarditis Virus (PMCV), Piscine Orthoreovirus (PRV subtypes 1,2 and 3) and Salmon gill pox virus.

The NGS platform is based on the immobilization of DNA/RNA onto a solid support, cyclic sequencing reaction through automated fluidic devices and detection of molecular events by imaging. NGS is a technique that is independent of the target sequence, but in order to obtain good coverage of the virus, it is important to analyse a sample that is rich in the viral pathogen with a limited presence of the host genetic material.

At sampling, it is recommended to collect tissues and organs for NGS analysis in case the first series of investigations does not provide conclusive results. For RNA sequencing, organ and tissue samples shall be stored in RNA later at -20°C or, preferably, at -80°C. For DNA sequencing, organ and tissue samples shall be stored frozen at -20 °C or, preferably, at -80 °C. In case an agent is isolated on cell culture, the cell supernatant and cell lysate shall be stored frozen at -20 °C or, preferably, at -80 °C.

14.4.5. Electron microscopy

Electron microscopy techniques can be used to morphologically characterize new pathogens. Electron microscopy has limited analytical sensitivity; therefore, suitable samples for this technique should originate from pathogens isolated on cell culture, or from field samples, where a high concentration of pure pathogen is expected.

14.5. General conclusions

Increased mortality should always be taken seriously and diagnostic investigations should be initiated as soon as possible. The following guidelines, based on this chapter, can be followed to perform a thorough investigation:

- Define the disease case highlighting all anamnestic features in the case description.
- Rule out non-infectious agents such as environmental conditions, toxic agents, etc.
- Rule out known pathogens with a focus on listed ones.
- Secure diagnostic samples from sick fish in an affected production unit (for example a tank or a cage), healthy fish in an affected production unit and healthy fish in a non-affected unit.
- Establish a chain of evidence where solid links are established between the symptomatology, necropsy findings, histopathological findings, microbiological tests and specific tests.
- Store material in different fixatives for future reference in case of inconclusive analyses, if the suspicion of an unknown disease agent is strong.

Keep in mind that it is of paramount importance, in order to conclude an identified pathogen as the cause of the disease and mortality, to corroborate the identification by means of different diagnostic techniques targeting different pathogen components (pathogen genome as well as antigens).

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