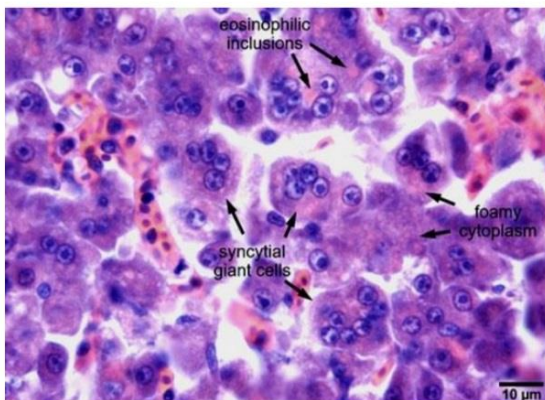
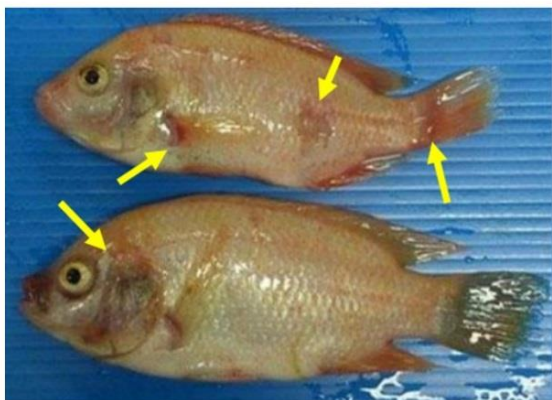
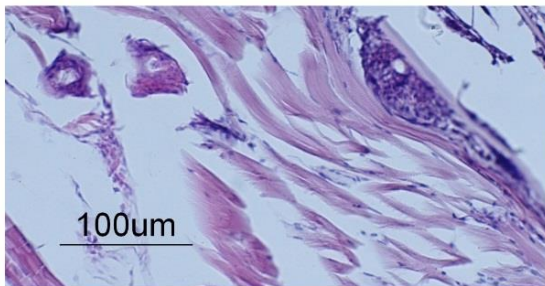
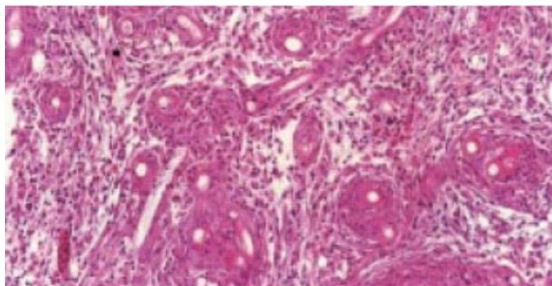


**UTF/ZAM/077/ZAM:
Technical Assistance to the Zambia Aquaculture Enterprise Development
Project (ZAEDP): Output 4: Improving Aquatic Animal Health**



Introduction

The FAO project UTF/ZAM/077/ZAM: Technical Assistance to the Zambia Aquaculture Enterprise Development Project (ZAEDP) funded by the African Development Bank has the overall objective of advancing the aquaculture subsector as a viable and inclusive business opportunity, through enhanced production and productivity, in order to improve the livelihoods of beneficiaries along the aquaculture value chain. These objectives would be achieved through: a) support and promotion of reliable access to quality input and output markets; b) competitively-priced risk sharing access to finance; and c) creation of an enabling infrastructure environment, which in turn is expected to facilitate the entry of new actors and expansion of business opportunities by the existing players in the industry. Specifically targeted fish farmers will improve their business and managerial skills and the quality of their productive systems in order to increase output, efficiently, while keeping fish prices affordable and meet growing demand from the lucrative domestic market.

The overall expected impact is increased incomes and living standards of households by inducing inclusive and sustainable aquaculture growth. The project will boost a worthwhile aquaculture subsector in Zambia in order to promote economic diversification, food security and sustainable employment generation, all of which are central priorities of the Government of Zambia. The development goal is to develop a domestic aquaculture subsector, which serves as a viable and inclusive business opportunity through enhanced production and productivity to improve the livelihoods of men and women beneficiaries along the aquaculture value chain.

There are five specific outputs:

1. Feed quality assurance capacity increased
2. Zambian finance institutions trained with respect to aquaculture and related risks
3. Food safety programme established
4. **Aquatic animal health management improved**
5. National aquaculture statistics database established and capacity increased

In view of the above three back-to-back events are being organized and will take place in Lusaka, Zambia from 11-17 October 2019, namely:

- Event 1: First meeting of the National Task Force on Aquatic Animal Health Management in Zambia (11-12 October 2019)
- Event 2: **Training Course on Development of an Active Surveillance for Epizootic ulcerative syndrome (EUS) and Tilapia lake virus (TiLV) using the FAO 12-point surveillance checklist (14-17 October 2019)**
- Event 3: National Stakeholder Consultation on ZAED: Improving Aquatic Animal Health (17 October 2019)

UTF/ZAM/077/ZAM: Technical Assistance to the Zambia Aquaculture Enterprise Development Project (ZAEDP): Output 4: Improving Aquatic Animal Health

Training Course on Development of an Active Surveillance for Epizootic ulcerative syndrome (EUS) and Tilapia lake virus (TiLV) using the FAO 12-point surveillance checklist

University of Zambia, Lusaka, 14-17 October 2019

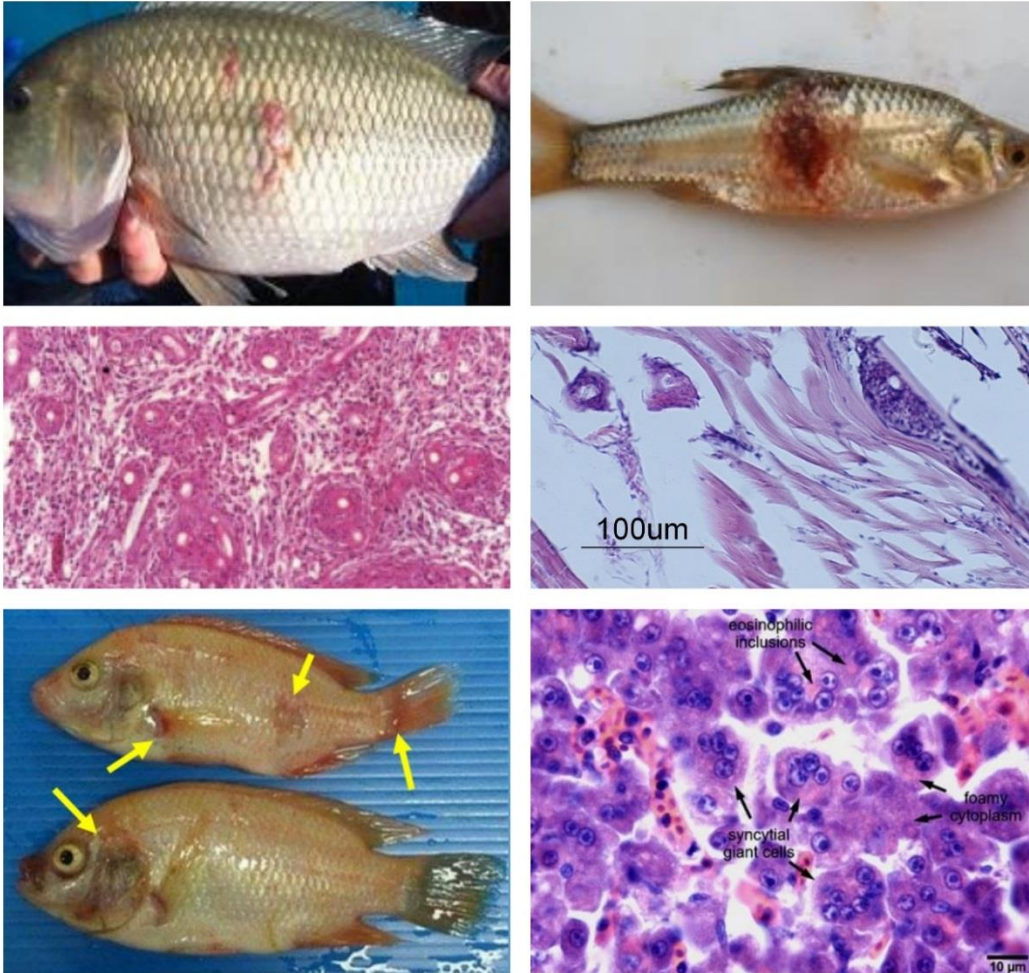


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SESSION 1: Introduction to the Training Workshop

Introduction to the ZAED project and the Training Workshop Four P's (M Reantaso)

The FAO project UTF/ZAM/077/ZAM: Technical Assistance to the Zambia Aquaculture Enterprise Development Project (ZAEDP) (ZAED) funded by the African Development Bank has the overall objective of advancing the aquaculture subsector as a viable and inclusive business opportunity, through enhanced production and productivity, in order to improve the livelihoods of beneficiaries along the aquaculture value chain. These objectives would be achieved through: a) support and promotion of reliable access to quality input and output markets; b) competitively-priced risk sharing access to finance; and c) creation of an enabling infrastructure environment, which in turn is expected to facilitate the entry of new actors and expansion of business opportunities by the existing players in the industry. Specifically targeted fish farmers will improve their business and managerial skills and the quality of their productive systems in order to increase output and efficiency, while keeping fish prices affordable and meet growing demand from the lucrative domestic market.

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There are five specific outputs, namely: (1) Feed quality assurance capacity increased; (2) Zambian finance institutions trained with respect to aquaculture and related risks; (3) Food safety programme established; (4) Aquatic animal health management improved ; and (5) National aquaculture statistics database established and capacity increased

The Training Course on Development of an Active Surveillance for Epizootic ulcerative syndrome (EUS) and Tilapia lake virus (TiLV) using the FAO 12-point surveillance checklist (for non-specialists) and its implementation pertains to Activity 4.5 (Implementation of some elements of the National Strategy (e.g. surveillance design and implementation for selected diseases (e.g. EUS, TiLV), diagnostics for selected diseases, farm-level biosecurity and border controls, emergency preparedness and contingency plans), one of 11 project deliverables under Output 4 (Aquatic animal health management improved).

Purpose. The purpose of this training course, being organized by FAO in collaboration with the Department of Fisheries and Veterinary Services and the University of Zambia, is to build EUS and TiLV capacity of trainees from Zambia through enhancing their knowledge on EUS and TiLV diagnostics (field and laboratory), surveillance, control and management, emergency preparedness and contingency plan within the context of Zambia aquaculture and wild fisheries.

Participation. The course will be participated by trainees representing the public and private sectors (Annex 2). Trainers include local experts (Dr Mwansa Songe and Dr Bernard Mudenda) and FAO experts (Dr Nihad Fejzic (Bosnia and Herzegovina), Dr Win Surachetpong (Thailand), Dr Kathy Tang-Nelson (USA) and Dr Melba Reantaso (FAO/HQ)) (Annex 1 for expert profiles).

Products. The expected outcomes of the course are: (i) Enhanced knowledge on all aspects of EUS and TiLV based on available information and experience; and (ii) Targeted surveillance plan for EUS and TiLV Action plan on TiLV prepared by trainees.

SESSION 2: Introduction to disease diagnostics and surveillance, EUS and TiLV

Factors in disease development (Snieszko circle) (M Reantaso)

Diseases in animal populations are rarely caused by a single factor, but represent the end result of a series of interactions between the host, environment, and presence of a pathogen or disease-inducing factor. This was captured by Snieszko (1974) in his diagrammatic representation of disease causality in finfish aquaculture, but applies equally to disease in any wild plant or animal population.



Snieszko circle: representation of relationship between host, pathogen and the environment in disease development.

Disease agents and factors are part of any ecosystem. Under a stable scenario ('healthy conditions'), pathogens usually exist in balance with their hosts, i.e., they persist via a small proportion of the host population, a process known as natural selection of the fittest. When an endemic (established) pathogen causes disease across a larger proportion of the population, either the environment has compromised the host species (temperature, food, habitat) or something else has impacted their disease tolerance (e.g., genetic mutation). In such instances, some infection-tolerant or resistant individuals with established immunity survive to rebuild the population. Destruction of the host population to the point of no recovery is rarely the result of an endemic pathogen.

Infectious agents generally cause the most devastating disease losses in populations that have no prior exposure to the infection and, therefore, no immunity. This situation is well documented for wild animals around the world as well as for human populations exposed to new disease agents. In the aquatic environment, where farmed production shares, or is only partially separated from, the wild environment, pathogens can pass freely between wild and farmed stocks. This is similar to insect-borne or air-borne pests and pathogens of terrestrial animals and plants.

Exotic pathogen introduction is usually a result of direct or indirect human intervention. A high risk of introduction is posed by movement of live animals for stocking (seed/larvae, broodstock or pre-market live-holding) to areas remote from the origin of the animals (Subasinghe *et al.*, 2001). Introductions can also be via contaminated equipment, boats or personnel moving from one water body, region or country to another. Although the animals being transported may show no signs of disease, they can be sub-clinical (healthy) carriers (disease-tolerant) and remain capable of shedding viable pathogens in the receiving waters; thereby exposing both wild and farmed animals to a new disease against

which they have no immune defense. This situation requires stringent surveillance and diagnostically-robust certification of freedom from high risk disease agents.

Aquatic Animal Health in Aquaculture Production

The complexity of farmed animal production in an aquatic ecosystem often makes distinction between disease caused by sub-optimal growing conditions (non-infectious physiological or physical stress) and pathogen-triggered disease obscure, at least in the early stages. Although aquaculture has been a local protein-production activity in many areas of the world for nearly as long as terrestrial animal farming, for many species it has only grown to an international production sector in the last 40-50 years.

With this expansion and diversification, many species are now farmed outside their natural geographic range. In addition, domestication of new species for aquaculture is one of the biggest production challenges for any farmer and is, usually, learned by trial and error. Domestication requires provision of habitat and feeds that replace wild conditions at a production cost that can be borne by the market value of the species being farmed. In addition, genetic selection of optimal producers under farmed conditions may take many generations to establish (disease tolerant, prime feed converters, and quality market product). The end result is what establishes both environmental and commercial sustainability of species used for aquaculture.

Role of diagnosis and diagnostic levels (I, II, III) (M Reantaso)

Diagnostics play two significant roles in aquatic animal health management and disease control:

1. **Screening** of healthy animals to ensure that they are not carrying subclinical infections by pathogens of concern. This is commonly conducted on samples of stocks or populations of aquatic animals destined for live transfer from one area or country to another, and provides protection on two fronts:
 - (a) it reduces the risk that animals are carrying few, if any, opportunistic agents that might proliferate during shipping, handling or change of environment; and
 - (b) it reduces the risk of resistant or tolerant animals transferring a significant pathogen to a population which may be susceptible to infection.
2. **Disease diagnosis** of animals showing signs of health deterioration (such as spawning failure, growth or behaviour) or clinical disease (deformities, morbidity or mortality). Accurate and rapid diagnosis is essential for applying appropriate and effective management measures.

Both applications of diagnostic analyses rely on a broad array of techniques ranging from gross observation to molecular probes and genetic sequencing. The choice of which tool to use for any diagnostic application relates to the *sensitivity* (ability to detect infections) and *specificity* (ability to distinguish one disease agent from another) of each technique; i.e., 'fit for use, fit for purpose'.

For surveillance of healthy animals, application of gross observation for clinical signs is, by definition, ineffective (i.e., not fit for purpose). Therefore, more sensitive and specific tools, such as PCR and related molecular techniques, are needed to detect low levels of infection in sub-clinical animals. Such techniques lack the ability to distinguish viable (infective) from non-viable (benign) infections, but act to provide an indication of risk that can be investigated further for the stock or population identified.

For diagnosis of a disease outbreak, a disease with a unique visible manifestation can be equally well diagnosed by the naked eye as it can by a genetic probe. Thus, investment in the latter technique for disease diagnosis would be cost ineffective, especially for an area or population known to be endemic for that disease. However, emergence of the disease in an area or population where it has not previously been observed warrants further investigation. In such a

situation, gross observations would be considered *presumptive*, but require further analyses to be confirmed and considered as a *conclusive* diagnosis.

Disease diagnosis in aquatic animal health management

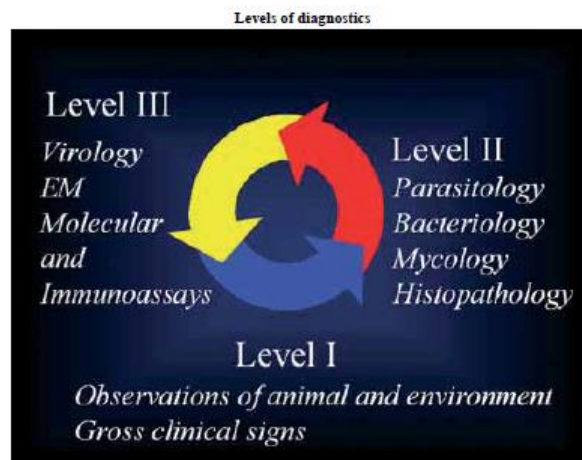
Accurate diagnosis of a disease can rarely be achieved by a single test, except in instances of regular occurrence and pathognomonic (disease-specific) clinical signs, as mentioned under #1 above. A presumptive diagnosis, indicating a strong likelihood of disease identification, is usually made with a single test but, frequently, needs to be reinforced by a second or third repeat or different test in order to be considered conclusive (100 percent certainty of causative pathogen). The importance of accurate diagnosis cannot be over-emphasized for aquaculture or any other medical scenario. Inaccurate diagnosis can lead to ineffective or excessive disease control measures – both of which are costly.

Diagnostic accuracy relies on a solid case-history. This starts with farm-level biosecurity measures, record-keeping and observations of feed-uptake, growth rates and behaviour. Abnormal observations may trigger the need for basic (routine) diagnostic tests for suspect recurrent diseases, or the need for more comprehensive testing; e.g., for 'new' observations or following introduction of new animals. As noted above, preliminary tests may produce conclusive results or trigger the need for more testing (repeat samples or different tests). Samples may need to be sent to laboratories with more specialized test equipment or experience with the disease of concern.

The evidence gathered from farm observations to the high-tech laboratory results are essential for accurate diagnosis, especially for previously unknown diseases (in an area/country or in aquaculture internationally). For aquatic animals, farm observations are especially important for contributing to accurate disease diagnosis. Water conditions, growth rates, records of sources of animals introduced, feed sources, etc., all contribute to directing the farmer or local authorities to the appropriate support for diagnostic analyses and confirmation.

None of the levels function in isolation, each one builds on the other, contributing valuable data and information for optimal diagnostic accuracy. Level 1 provides the foundation and is the basis of for accurate interpretation of results obtained from Levels II and III laboratory findings.

Levels of Diagnosis



Level I (farm/production site observations, record-keeping and health management) is strongly emphasized throughout the *Asian Diagnostic Guide* as this forms the basis for accurate results from Levels II and III diagnostic analyses.

Level II includes the equipment and experience to undertake analyses that can detect and/or identify a range of pathogens. Level II laboratories can do parasitology, histopathology, bacteriology and mycology examinations, and are generally-speaking experienced with endemic and opportunistic disease agents in their area, region or country.

Level III diagnostics encompass techniques that target a specialized pathogen or group of pathogens or require highly specialized equipment. These laboratories require significant capital and training investment. Some are accredited by national or international authorities as 'reference laboratories' for specific pathogens and are used to confirm a new detection or identify a new pathogen that requires more investigation. Immunology and biomolecular techniques are included in Level III, although field kits are now available for farm or pond-side use (Level I) as well as in microbiology or histology laboratories (Level II) for some pathogens (Walker and Subasinghe, 2000). Such technology transfer is enhancing diagnostic capability and, with solid quality control and field validation, more Level III technology will continue to become field accessible.

One of the most important aspects of the effectiveness of the three diagnostic Levels is ensuring that Level I observers have access to, and know how to contact, Levels II and III support (and at what cost). Level III diagnostic support is usually based on referrals so has little input on field conditions. They, or the referring authority, need to connect field observations to Level III results to ensure that all align with the disease situation being investigated.

Level III laboratories are highly specialized and usually develop in areas where serious disease challenges are recurrent and have warranted research essential to the development of disease-specific expertise and diagnostic technologies. Many such laboratories are accredited nationally or by the OIE as 'Reference Laboratories' (OIE, 2017). These laboratories can also be used to confirm disease-freedom to reinforce national certification for import-export purposes.

It is very important to let authorities (local, regional or national) know of any samples being sent for outside the country for confirmatory testing for a new, or presumptive exotic, pathogen detection in the country. This is because a confirmatory finding can trigger market closures, impacting the whole country versus the farm or area where the infection was detected. A trade closure with no national authority preparedness places all exports at jeopardy of loss of market access. In addition, OIE reference laboratories are obliged to inform the Chief Veterinary Officer (CVO) of the country when they confirm the diagnosis of any OIE notifiable disease (or a pathogen of a new and potentially significant infectious disease). Ensuring the CVO is aware of the presumptive diagnosis allows the national aquatic animal health authority to work with regional/local authorities on interim disease control measures that can be used to show diligence and disease response competence if the disease agent is confirmed.

Where there are few aquatic animal health problems, there is little incentive to invest in diagnostic laboratory capacity. Strong links with Level II and/or III diagnostic expertise is a good precautionary measure for general health surveillance, health certification of import stock, and to reduce the risk of disease introduction into disease-free areas.

The importance of diagnostic accuracy and understanding of sensitivity/specificity of the tools applied to a disease outbreak situation cannot be over-emphasized. Rapid response is essential and many diagnostic techniques can take days or weeks to complete. An immediate control measure is isolation of the affected aquatic animal population(s) pending confirmation of the disease agent and/or delineation of the affected area, farms and/or network facilities. This stops movement of any animals away from the affected sites or farms and imposes stringent biosecurity measures to personnel and equipment. In some instances, stock destruction may be called for, so rapid and accurate confirmation of the disease agent is critical.

In many situations, some animals may show clinical signs of infection. These are usually the easiest samples for detection and identification of the infectious agent. However, other animals on the farm, on neighbouring farms, or in surrounding waters may appear healthy. These require more sensitive analyses (tests that can detect very low levels of infection by the causative agent) to determine if these are truly free of the disease agent and need protection from disease spread.

In summary, inclusion of all levels of diagnostic observations and applying a matrix of results to decision-making gives the most solid foundation possible for risk analysis at the farm, regional, national and international levels of aquaculture production, as well as for effective disease response.

What is epidemiology? What is surveillance? (N. Fejzic)

Epidemiology is the study of the distribution and determinants of health-related states or events (including disease), and the application of this study to the control of diseases and other health problems. Various methods can be used to carry out epidemiological investigations: surveillance and descriptive studies can be used to study distribution; analytical studies are used to study determinants. Surveillance was defined as all regular activities aimed at ascertaining the health status of a given population with the aim of early detection and control of animal diseases of importance to national economies, food security and trade. Monitoring on the other hand was defined as all activities aimed at detecting changes in the epidemiological parameters of a specified disease. Both definitions were taken from FAO Manual of livestock disease surveillance and information system. Besides laboratory diagnostic procedures, which start from individual diseased animals, and continue investigation of its organs, tissues and all the way to cell and molecular level in order to determine disease cause, there are other ways to understand disease causality. Epidemiologists start with the animal, and move upwards to the level of pond, farm, area/zone, etc. At each level, an epidemiologist is trying to understand the patterns of disease in order to understand what component factors may be involved in causing the disease. There are several tools that epidemiologists use to understand patterns of disease in populations. One example is the use of outbreak investigations or case studies. Surveillance is another tool for understanding patterns of disease in the population.

In order to differentiate disease and health status between individual animals or any other units of observation, it is important to first have a clear idea of what we consider by a disease. Some diseases are caused by a known pathogen, while others have multiple causes sometimes involving pathogen, but also environmental or management factors. In general disease status can be defined as any abnormality of structure or function. In epidemiology disease and health are categorized based on case definition which includes list of criteria (i.e. limits on time, susceptible host, place, as well as specific combination of symptoms and test results). The reason for recognizing disease is to prevent it, control it, or minimize its negative effects. Besides knowing which individuals or units of observation are considered diseased, we must understand something about the cause of the disease. More information about infectious agents of aquatic animal diseases are provided in OIE Manual of diagnostic tests for aquatic animals. However, in many cases, it may be very hard to identify an infectious agent causing a particular disease problem. Nevertheless, if other factors or component disease causes can be identified, it may well be possible to control the problem without ever knowing exactly what the agent was.

Importance of surveillance in aquatic animal health management (N Fejzic)

Globally, capture and culture fisheries contribute significantly towards food security, poverty alleviation, economic development and supporting livelihoods. Aquaculture and global trade continue to intensify and expand, while aquatic animal diseases are a major risk and a primary constraint to the growth of the aquaculture sector in many countries and regions. Over the years instruments have been developed, by the international development and standard setting organisations (such as FAO and OIE) to help national governments to protect themselves from diseases without setting up unjustified sanitary barriers. The main normative documents produced by the OIE for aquatic animals are the Aquatic Animal Health Code (Aquatic Code) and the Manual of Diagnostic Tests for Aquatic Animals (Aquatic Manual), as well as the FAO Code of Conduct for Responsible Fisheries supplemented by numerous technical guidelines (references on the Code and manual as FAO code). These documents provide details of health measures to be used by the veterinary or other competent authorities to minimise introduction and spread of major aquatic pathogens in a systematic, scientifically based, transparent and internationally recognized manner. Accounting for the wide diversity

of aquaculture production and individual country needs, National aquatic animal health strategies (including biosecurity) need to be developed for every country as basic framework for implementation of health management strategy covering specific issues to be addressed at the farm/state/national level. Surveillance is an important element of a national biosecurity and aquatic animal health management strategy, which needs to be implemented in the context of the aquatic and aquaculture environments in order to comprehensively account for all risks of disease introduction and/or spread.

Introduction to EUS (M Songe/B Mudenda HangOmbe)

Epizootic ulcerative syndrome (EUS) is a severely infectious disease of fish that is known to affect more than 100 types of wild, farmed freshwater and estuarine fish, and has historically caused serious losses in the aquaculture industry. OIE has confirmed that EUS is a seasonal epidemic caused by infection with the highly invasive, deeply penetrating pathogenic oomycete *Aphanomyces invadans* or *Aphanomyces piscicida*, and is listed among those diseases that must be notified.

EUS has swept across many countries globally. This has caused significant losses of income to fisheries and fish farmers and negative biodiversity and social impact. The economic impact of this disease is huge. It has caused an estimated loss of USD 110 million only in few countries of Asia-Pacific region during the late 1980s and early 1990s. Clinical EUS has not been reported from Europe so far, but *A. invadans* has been detected in imported ornamental fish species from different non-European countries.

The first report of the disease came from Japan, where it is known as mycotic granulomatosis, in 1971. In Australia, where it primarily affected estuarine mullet, it was called Red Spot Disease (RSD). Since the early 1980s, EUS has spread westwards across the South-East and South Asia, affecting over 100 species of freshwater fish. The disease was first reported in Bangladesh in 1988. Outbreaks of Ulcerative disease in menhaden, *Brevoortia tyrannus*, in the United States of America (USA) have been shown to be very similar to EUS in Asia. The first case of EUS in the USA occurred in 1978. The pattern of spread between and within countries was consistent with progressive dissemination of a single infectious agent. On the basis of findings from studies conducted by Lilley and others, the authors suggested that *A. invadans* achieved its colonisation of Australia, Asia and by implication USA, in one relatively rapid episode, consistent with reports of outbreak occurrence.

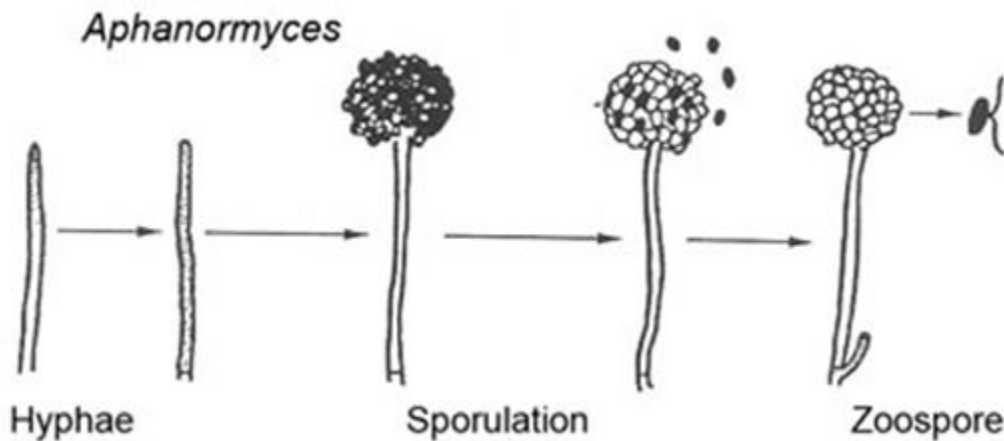
The first outbreak of EUS in 2007 in Zambia and other countries sharing the Zambezi River decimated fish populations until an enzootic stability status was attained on the Zambezi River. Since then, the disease has spread to other areas. For instance, in Zambia EUS has recently been recorded in the Bangweulu swamps, an inland delta, in the north of Zambia, an area previously believed to be free of the oomycete. The outbreak was of great significance as the Bangweulu swamps drain into the Congo River in neighbouring Democratic Republic of Congo, Africa's largest drainage system with an extensive and diverse fish fauna previously unaffected by EUS.

At present, Red Spot Disease, Mycotic Granulomatosis, Ulcerative Mycosis and Epizootic Granulomatous Aphanomycosis are all synonyms for EUS.

Pathogenesis of EUS. *A. invadans* has been shown to be slow-growing and thermo-labile in culture. The presence of *A. invadans* is accompanied by an intense granulomatous inflammatory response. Once established in the tissues of susceptible fish, *A. invadans* migrates towards the central nervous system, and then throughout the body, producing proteolytic enzymes which destroy muscle and other tissues. This species occurs worldwide and infects both wild and cultured fish, often leading to mass fish mortality. In the United Kingdom, Lilley and Roberts provided convincing evidence that *A. invadans*, and not one or more other pathogens, is responsible for much of the characteristic pathology of EUS. They injected zoospores from 58 oomycete isolates intramuscularly into snakehead fish, *Channa striata*. These oomycetes comprised of: *Aphanomyces* strains isolated in Asian countries and Australia from EUS-affected fish; saprophytic *Aphanomyces*, *Achlya* and *Saprolegnia* spp. from infected waters; and oomycetes involved in other

diseases of aquatic animals. Only the *Aphanomyces* strains isolated from fish affected by EUS, RSD or MG were able to grow invasively through the fish muscle and produce the distinctive EUS lesions. The snakehead-pathogenic strains were further distinguished from all the other oomycetes under comparison by their characteristic temperature-growth profile and inability to grow on certain selective media.

A. invadans is aseptate and produces two zoospore forms, the secondary form being free-swimming and laterally biflagellate. No sexual reproductive structures have been observed in any of the isolates from EUS, MG or RSD outbreaks. The lack of sexual structures is considered to be a particularly common phenomenon among the more pathogenic members of the Saprolegniaceae. *A. invadans* has a life cycle that consists of three different stages: hyphae, zoospore, and cyst. Changes in the aquatic environment (such as a change in salinity or water temperature) cause *A. invadans* to undergo asexual reproduction. During sporulation (asexual reproduction) thousands of swimming zoospores are released into the water. Infection occurs when these motile spores are attracted to the skin of fish. They penetrate the fish skin and germinate, forming oomycete hyphae. The latter invade widely into the surrounding skin and deeply into underlying muscle tissues, resulting in extensive, bloody ulceration and destruction of tissues. Initially, the lesions may be characterised by the appearance of raised areas of induration and erythema. Subsequent skin erosion results in the formation of ulcerative lesions on the body. Varying sizes of ulcerative lesions may progress to necrotizing dermatitis resulting in deep dermal ulcers. If a host is not found, the zoospores become cysts and sink to the mud or sediment.



Life cycle of *A. invadans* (Lilley *et al.*, 1998).



Life cycle of *A. invadans* showing hyphae, sporulation and zoospore stages.

Pathogenicity studies of *A. invadans* in *Oreochromis niloticus*, *O. andersonii* and *Barbus paludinosus* in Zambia reviewed that *O. niloticus* does not succumb to infection with *A. invadans*, even following experimental infection in the lab. In the same experiment, *O. andersonii* and *Barbus paludinosus* showed severe clinical disease and, through re-isolation of *A. invadans* from infected tissue, Koch's postulates were confirmed.

Risk factors associated with outbreaks of EUS. *A. invadans* is a slow-growing monoclonal pathogenic organism which, if provided with suitable conditions, is allowed to invade the tissues of susceptible fish. Initiating factors which facilitate the entry of the oomycete are varied. In some locations infection is predisposed by cooler water temperatures, in others higher temperatures appear to trigger it. EUS has often occurred after periods of heavy rain.

From laboratory observations, low salinities appear crucial to the transmission of the pathogen. Environmental parameters have also been studied. These abiotic factors are believed to cause sublethal stress to the fish, initiating disease outbreaks. Potential causes of stressful environmental conditions include: temperature, eutrophication, sewage, metabolic products of fishes, industrial pollution and pesticides. The quality of water also appears to be significant from an aetiological point of view. Parameters like salinity, alkalinity, temperature, hardness and chloride concentration (many of which are seasonally variable) are known to predispose fish to attacks of EUS. Infected fish shows signs of improvement when transferred to clean freshwater ponds.

Aphanomyces. invadans requires some predisposing condition of the host, such as debilitation or breach of the normal mucosal barriers along with favorable growth conditions before they can become established. Such conditions would include compromise of the animal's normal immune status (i.e., systemic and/or mucosal immunity). Some tank trials have been used to show that healthy, intact fish in aquaria exposed to *A. invadans* zoospores in water would not develop EUS lesions and that prior damage to skin was necessary before lesions could be induced in fish.

Summary. EUS is caused by a fungal like organism, the oomycete *A. invadans*, and can have devastating social, economic and biodiversity impacts on affected communities. Poor environmental conditions predispose susceptible fish species to infection. *O. niloticus* has previously been shown to be resistant to infection with *A. invadans*. It must therefore be handled with care when co-cultured with susceptible fish species as it may probably harbor the pathogen and hence be a 'carrier' of the disease.

Introduction to TiLV (W Surachetpong/K Tang-Nelson)

One of the most important disease in tilapia is an emerging viral diseases named tilapia lake virus disease (TiLVD). Tilapia lake virus (TiLV) has been shown to associate with high mortality in tilapia such as Tilapia one month mortality syndrome (TOMMS) in Thailand, and Summer mortality syndrome in Egypt. In 2015, massive mortality of tilapia has been observed in Thailand after fish (at 2–30 g) are transferred from hatchery to the grow out cages. During 3–6 week after the transfer, fish start clinical signs of infection and mortality with rapid mortality in 10–14 days. The cumulative mortality range between 20–90 percent. The gross signs of infection include skin erosion, exophthalmos, skin redness, abdominal distension, and anemia. Various bacteria, and parasites are isolated and detected in tilapia one month mortality syndrome including *Aeromonas*, *Streptococcus* and *Flavobacterium*. The prediction of economic impact of TOMMS for tilapia production in Thailand include the impact on mortality of fry and fingerlings making the fry shortage accounting for USD 2–4 million. High mortality in red tilapia than Nile tilapia caused some producers to change the production from red tilapia to Nile tilapia. Notably, the farmer can sell survived fingerlings at 10–30 cents more than fish that never pass the disease. In production unit, if the disease occurs, the consequence is to eliminate positive broodstock population. Finally, the cost of removing of sick and dead animals affect the production of many farmers.

In 2014, there is a study from Israel reporting the identification of a new RNA virus that mainly affect tilapia in Lake Galilee of Israel. High morbidity and mortality could be observed since 2009 in wild and farm raised tilapia causing significant impact on tilapia production in the area. Later, the research team identified a new RNA virus using molecular technique and viral isolation in primary and continuous cell lines. Laboratory challenges using intraperitoneal injection and cohabitation of sick fish with susceptible fish revealed high mortality within 7–14 days. At the same time, a research team in Ecuador investigated a problem of high mortality in juvenile tilapia with clinical signs of ascites, exophthalmia. The histopathology of liver revealed a distinct lesion of liver necrosis and syncytial cell formation. The disease has been named syncytial hepatitis of farmed tilapia. As the case description and clinical appearance of diseased fish in

Thailand, we suspected that TiLV may responsible for mass mortality of red and Nile tilapia in Thailand. The RT-PCR protocol confirmed that more than 60 percent of high mortality in tilapia associated with TiLV. The virus was isolated in E-11 cells (derivative of SSN-1 clone isolated from snakehead fish). In Thailand, investigation of 32 outbreaks of high mortality in tilapia revealed that 22 outbreaks are positive to TiLV. Experimental infection of red tilapia and Nile tilapia showed cumulative mortality range between 60-80 percent.

Current information on TiLV. TiLV is a single stranded RNA envelope virus. The virus shares some characteristics with the virus in family Orthomyxoviridae (Orthomyxo-like virus). The virus is mainly affecting tilapia and closely related species and giant gourami while most fish species are resilient to TiLV. There is no report of TiLV infection in human or other animals. To date, TiLV has not been listed on the OIE list disease due to the incomplete validation of diagnostic assay. To control the spread of virus in tilapia farm, strict biosecurity should be implemented to reduce the impact of disease. There are TiLV confirmations in at least 15 countries in 3 continents either by official report to OIE or international publications. Mass mortality of tilapia associated with TiLV has been observed in Malaysia, Indonesia and Peru. The virus can infect tilapia at all sizes from 10 days after hatching, fries, juveniles, fingerlings, and broodstock.

Farm experience of TiLV outbreak often find high mortality above 2–3 percent for 2–3 consecutive days with fish showing signs of swimming at the water surface, skin redness and erosion, pale body color, abdominal swelling, and exophthalmos. Coinfection of bacteria, parasite and TiLV are commonly found during the outbreaks. The bacteria in genus *Aeromonas* such as *A. hydrophila* are isolated from 50 percent of TiLV infected fish in Egypt. In addition to the detection of TiLV in clinically affected fish, a study in Tanzania and Uganda showed that TiLV could be detected in healthy fish (14.6 percent) using RT-PCR assay.

The transmission of TiLV between fish is primarily via horizontal route and high levels of virus are shed from infected fish through mucus. Additionally, vertical transmission via infected brood stock to the offspring have been documented. Upon entry, the virus spread through various internal organs of fish including gills, spleen, anterior kidney, liver and shed via mucus causing the disease outbreak in fish population.

Summary. Tilapia Lake Virus is an emerging viral disease associated with high morbidity and mortality in tilapia. To date, TiLV has been reported in 15 countries. Multiple infections of TiLV, bacteria and parasites are commonly found in Summer mortality, or One month mortality syndrome in tilapia. Strict biosecurity and development of a vaccine will be important tools to reduce the impact of TiLV to tilapia aquaculture.

SESSION 3: Development of an active EUS and TiLV surveillance using the FAO 12-point surveillance checklist (for non-specialists and for developing countries)

Introduction to the FAO 12-point surveillance checklist (M Reantaso)

Government services and officers responsible for aquaculture health management in developing countries often have limited formal education in epidemiological approaches to disease control. Conversely, the Aquatic animal health code of the World Organization for Animal Health (OIE) (OIE, 2018) request that member countries provide information regarding its aquatic animal health status, allowing transparency so that planning, surveillance activities, analysis, and availability of data and information are maintained at all times in compliance with prescribed standards. In such situations, development of country's capacity to apply surveillance and survey programmes is challenging, particularly when the resources needed for training and implementation of surveillance programme are limited. Knowledge of the fish species biology, aquaculture practices, interaction with wild aquatic species and other aspects of aquaculture health management are essential for an interdisciplinary approach to aquatic disease control. An additional problem is that monitoring and surveillance activities for aquatic animal diseases conducted by governmental offices often remain unpublished. In addition, there is limited transfer of field and scientific information between developed and developing country aquatic health surveillance systems.

The development of the FAO 12-point surveillance checklist is in response to requests from FAO member countries for detailed guidance on the design and implementation of surveillance programs for aquatic diseases that is practically applicable for developing countries and to be implemented by non-specialists, i.e. government personnel and extension officers with no formal training on epidemiology but building on their skills in practical aquaculture and basic knowledge on aquatic animal health. Thus, this 12-point checklist have been developed by FAO in collaboration with country proponents of several countries participating in FAO projects. The guidance for the design and implementation of an active surveillance for targeted diseases is work in progress and continuously being updated as experience and knowledge in field application are gained. The ultimate objective is to achieve a practical but methodological (step-by-step) education for non-specialists in developing countries facing common technical, environmental, infrastructural and financial challenges. The main references used in the development of the checklist are those of Cameron (2002), Subasinghe, McGladdery and Hill (2004) and Corsin *et al.* (2009) to ensure that the main elements of any aquatic animal surveillance programme are captured.

Table 1 below presents the 12-point surveillance checklist as steps (nos. 1–12), with a description of each step and the criteria or important considerations in each of the 12 steps.

Table 1. 12-point surveillance checklist that contains the step description and requirements (criteria)

Step	Description	Criteria
1	Hazard/Disease scenario setting	<p>Selection of disease of concern will depend on country proponents; ideally it should be included in a country's National Pathogen List. It can fall under any of the following categories:</p> <ul style="list-style-type: none"> • exotic: not present in the country, e.g. OIE-listed diseases of relevant aquaculture species of a country • endemic: diseases that are commonly affecting cultured species (i.e. production-related diseases, mostly bacterial or parasitic diseases) • emerging: either known (spreading to new geographical areas or affecting new susceptible species) or unknown and previously undiagnosed diseases

2	Defining data collection objective	<ul style="list-style-type: none"> • Set with respect to disease • Set with respect to disease presence • Set with respect to level of certification • Set with respect to timeframe
3	Definition of population	<ul style="list-style-type: none"> • Includes definition of the population of interest • Includes definition of the targeted population • Includes definition of the study population (population used for sampling) • 3.a. Inclusion criteria are set and described • 3.b Exclusion criteria are set and described
4	Clustering of disease	<ul style="list-style-type: none"> • Clustering effect of disease is considered and described • 4.a. Clustering effect of disease is accounted in sampling/survey design and data analysis
5	Case definition/outbreak definition	<ul style="list-style-type: none"> • Case/outbreak definition are included • 5.a. Clinical • 5.b. Laboratory • 5.c. Epidemiological
6	Diagnostics/Field and laboratory tests	<ul style="list-style-type: none"> • List and description of tests used (procedures, interpretation of results, Se/Sp) • List of laboratories included
7	Study design and sampling	<ul style="list-style-type: none"> • Used/described sampling frame • 7.a. Described sampling method • 7.b Defined sampling units • 7.c.Explained consideration regarding sample size • Describe tissues/fluids used as sampling material • 7.a.1. Describe sample selection process
8	Data flow and management	<ul style="list-style-type: none"> • Data forms included • Data base (design and management) • 8.a. Compatibility of data throughout the collection/analysis process and transparency • 8.b. Consistency, quality and precision of data
9	Data analysis and methodology	<ul style="list-style-type: none"> • Survey design described • Risk assessment used and described • Methods of data analysis described
10	Validation and quality assurance	<ul style="list-style-type: none"> • Done by statistical estimation of the level of confidence (Se of surveillance program) • Done by pilot trial • Done by expert/external evaluation (peer-review) • Audit and corrective measures
11	Human and financial resources and logistics requirements	Included and described (e.g. personnel, cost of sampling, cost of laboratory tests, analysis of data, etc.)
12	Putting surveillance in the bigger picture (NSAAH and PMP/AB)	Surveillance as an essential component of aquatic animal health/aquatic biosecurity strategies, aquatic animal health protection programmes or disease control plans

The below section describes in detail each step of the checklist, some key elements and examples.

Checklist #1: Hazard/Disease scenario setting (M Reantaso)

Oftentimes, it is quite a challenge to start the process of designing a surveillance programme. Scenario setting is a good first step that involves understanding the health status of a specific pathogen or disease in a country. The three likely scenarios are:

- Scenario 1: Infected: the aquaculture species is infected with the pathogen or disease of concern as supported by one or more cases reported during the two previous years
- Scenario 2: Considered free: the country did not have any reported cases in previous surveillance activities
- Scenario 3: Unknown status: (1) there are no reported cases and no previous surveillance activities; (2) there are reports of cases (grey and scientific literature) and/or information from the producer sector but not officially reported by the Competent Authority to OIE (in case of OIE-listed disease). In all cases, the country is considered at risk.

The selection of the scenario will assist in drawing the next step, i.e. defining the surveillance objective.

Checklist #2: Defining data collection objective (M Reantaso)

2	Defining data collection objective	<ul style="list-style-type: none"> • Set with respect to disease • Set with respect to disease presence • Set with respect to level of certification • Set with respect to timeframe
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The objective of surveillance is strongly associated with disease of concern and the status of the disease in a country, the level of certification and the time-frame. Defining a clear objective is a very important step as the components and other requirements and activities of the surveillance system will be determined based on the set objective. The table below shows some examples of the aim of the surveillance based on the health status scenario; the disease of concern is Epizootic ulcerative syndrome (EUS).

Table 2: Setting the objective/purpose of the EUS surveillance according to different health status scenarios: examples.

EUS surveillance scenario	Objective/purpose of EUS surveillance	Countries
Infected country (one or more EUS cases reported in previous two years)	To establish frequency of the EUS at national level in wild and farmed populations for a period of one year To identify possible risk factors for EUS spread for the purpose of developing more targeted disease control program To establish a transparent reporting system (according to OIE requirements)	Zambia Zimbabwe
Considered free (no reported cases in previous surveillance activities)	To confirm present status of EUS in country To secure early detection of EUS	Malawi Mozambique
Unknown status (no reported cases and no previous surveillance activities, however considered at risk)	To investigate presence/absence of EUS in wild and farmed fish To secure early detection of EUS	Botswana Namibia

Table 3: Setting the objective/purpose of the TiLV surveillance according to different health status scenarios: examples.

TiLV surveillance scenario	Objective/purpose of EUS surveillance	Countries
Infected country (one or more TiLV cases reported in previous two years)	To establish frequency of the TiLV at national level in wild and farmed populations for a period of one year To identify possible risk factors for EUS spread for the purpose of developing more targeted disease control program To establish a transparent reporting system (according to OIE requirements)	
Considered free (no reported cases in previous surveillance activities)	To confirm present status of TiLV in country To secure early detection of TiLV	Zambia (?)
Unknown status (no reported cases and no previous surveillance activities, however considered at risk)	To investigate presence/absence of TiLV in wild and farmed fish To secure early detection of TiLV	Zambia (?)

Checklist #3: Definition of population (N Fejzic)

3	Definition of population	<ul style="list-style-type: none"> • Includes definition of the population of interest • Includes definition of the targeted population • Includes definition of the study population (population used for sampling) • 3.a. Inclusion criteria are set and described • 3.b Exclusion criteria are set and described
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Existence of population data is the most important variable in designing of surveillance and interpretation of its results. If nonexistent, this step will require the listing of susceptible wild fish species, present or likely presented in rivers, lakes and other water bodies as well as a list of farmed fish species in order to determine population of interest, targeted population and study population in accordance to achieve surveillance objectives. In addition, the geographical distribution of rivers, lakes and water bodies should be mapped and organized into zones based on water flow and/or the provinces or districts and neighbouring counties involved. Population data and information should be mapped accordingly (i.e. which species inhabits which water bodies). These activities include compiling a list of farm/ponds, which is needed to establish a sampling framework. Only from such information, sampling size may be determined. However, in developing countries population data often doesn't exist, so the surveillance planner and developer must rely on estimation and approximation and such approach will limit results gained through surveillance. Merging targeted/study populations data with relevant spatial and environmental data may as well be useful for understanding/recognizing risk factors (clustering of disease) leading towards targeted and more effective early detection and control activities. Irrespective of whether a country is known to be infected, or may be uninfected despite earlier surveillance, the population to be surveyed should be susceptible fish species (juveniles and adults), and all farmed species, except in known infected countries, in which wild populations should also be included.

Checklist #4: Clustering of disease (N. Fejzic)

4	Clustering of disease	<ul style="list-style-type: none"> • Clustering effect of disease is considered and described • 4.a. Clustering effect of disease is accounted in sampling/survey design and data analysis
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Cases of disease, especially infections, occur in clusters either through the time or in space. For many diseases, contributing factors related to a host (i.e. susceptibility), environment (i.e. season, climate, contacts) and agent (virulence, survivability, host specificity etc.) are more or less documented. Therefore, if information on aquaculture population, environment, farming practices and animal movements/contacts is known, it is possible to predict when and where more than expected disease occurrence will emerge (disease clusters), and direct detection and control activities accordingly (i.e. risk based surveillance). Even though not all disease clusters represent real outbreaks, keeping track of the agglomeration of cases, symptoms, mortality etc. is very efficient tool for early detection and warning (Rodriguez-Prieto *et al.*, 2015). Considering EUS surveillance, it is important to take into account that disease occurs mostly at water temperatures ranging from 18–22 C and after periods of heavy rainfall. This environmental condition has to be considered during the sampling period.

Checklist #5: Case definition/outbreak definition (N Fejzic)

5	Case definition/outbreak definition	<ul style="list-style-type: none"> • Case/outbreak definition are included • 5.a. Clinical • 5.b. Laboratory • 5.c. Epidemiological
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The purpose of a case definition is to assure that the surveillance will focus on the disease of concern and not any other disease showing similar clinical signs. Case/outbreak definition might include clinical, laboratory and epidemiological determinants of disease in consideration. On the other hand, strong consideration in making the case definition is to ensure sufficient surveillance system sensitivity (ability of the system to recognise disease in early stages of onset/introduction).

The following case definition might be adopted for purpose of EUS surveillance:

- Suspect EUS case: A fish showing red spots, ulcers or lesions similar to the ones associated with EUS followed by positive finding of fungal hyphae in a tissue squash.
- Suspect EUS location/farm: A location/farm where one or more suspect EUS fish have been found.
- Confirmed EUS case: Histopathology or fungal isolation or PCR.
- Confirmed EUS location/farm: A location/farm where a case of EUS has been confirmed.

TiLV is not OIE listed aquatic disease, so for purpose of this surveillance following model might be used:

- (suspected) A tilapia farming system in which the farmer has observed during the previous and ongoing production cycles sudden mortalities and/or clinical signs such as skin redness/erosion or eyes protrusion/ruptured/cloudiness or abdomen swollen or scale protrusion/loss, attributable to the presence of TiLV (e.g. farmer answer “yes” to the question whether TiLV has occurred or not in the farm of interest).
- (confirmed) Upon the collection of 30 moribund or sick fish samples, TiLV is confirmed by a positive test result using PCR and the detection of histopathological signs of TiLV.

Checklist #6: Diagnostics/Field and laboratory tests (M Songe/B Mudenda HangOmbe for EUS; W W Surachetpong/K Tang-Nelson for TiLV)

6	Diagnostics/Field and laboratory tests	<ul style="list-style-type: none"> • List and description of tests used (procedures, interpretation of results, Se/Sp) • List of laboratories included
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EUS

Epizootic ulcerative syndrome or EUS is an infection caused by an oomycete fungi known as *Aphanomyces invadans* or *A. piscicida*. It is an epizootic condition affecting wild and farmed freshwater and estuarine finfish. EUS is also known by other names such as red spot disease (RSD), mycotic granulomatosis (MG), ulcerative mycosis (UM) and epizootic granulomatous aphanomycosis (EGA) following the characteristic ugly lesions that develop during infection. Infection in fish occurs when motile spores of the fungi *Aphanomyces invadans* in the water or other carriers/ vectors are attracted to the skin of the fish. These spores will penetrate the skin and germinate, forming fungal filaments or hyphae. The hyphae invade into the surrounding areas of the skin and deep into the underlying muscle tissues, resulting in extensive ulceration and destruction of tissues.

Diagnosis of EUS disease. Examination of sick and dead fish with ulcerations will provide a presumptive diagnosis of EUS. Basically, the presumptive diagnosis of EUS can be based on gross appearance of open skin ulcers and the observation of aseptate hyphae in squashed preparations of the muscle underlying gross lesions. Confirmatory diagnosis requires demonstration of the typical granulomatous inflammation around invasive hyphae, isolation of *Aphanomyces invadans* from the underlying muscle and the demonstration of the fungal DNA in the affected tissues using PCR.

Level I: Gross signs and behaviour. EUS affected fish will show various clinical signs depending on host species and degree of infection. Initial lesions appear as red spots that may be observed on the body surface, head, operculum or caudal peduncle. Large red or grey shallow ulcers, often with a brown necrosis, are observed in the later stages with large superficial lesions (open dermal ulcerative lesions) occurring on the flank or dorsum. In the early stage of the disease signs include loss of appetite and fish become darker. Infected fish may float near the surface of the water, and become hyperactive with a very jerky pattern of movement. In some cases, the fish may be constantly appearing on the surface of water exhibiting gasping abnormality.

Level II: Histopathology: This technique involves the demonstration of the fungal structures in the tissues of the infected fish. This can be done under the following procedures:

a. Rapid squash muscle preparation

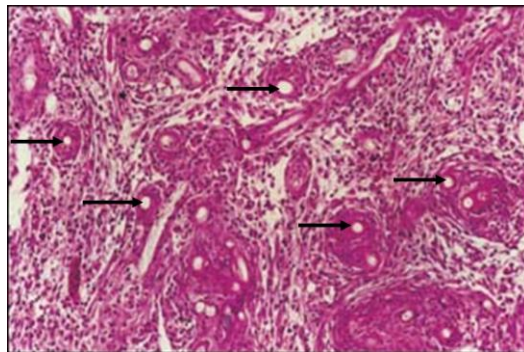
Presumptive diagnosis of EUS in susceptible fish showing dermal lesions can be made by demonstrating aseptate hyphae (12–30 µm in diameter) in squash preparations of the muscle underlying the visible lesion. This can be achieved using a thin piece of muscle squashed between two glass plates or microscope slides and examined using a light or dissecting microscope under field conditions. This procedure will demonstrate fungal hyphae in the lesions.



Hyphae in the squash preparation from the EUS lesion shown by the Arrow

b. Tissue histopathology

This is a confirmatory diagnostic test and requires histological demonstration of typical granulomas and invasive hyphae using haematoxylin and eosin or other fungus stains such as Grocott's staining. Early EUS lesions show shallow haemorrhagic dermatitis with no obvious fungal involvement. Later lesions demonstrate *A. invadans* hyphae penetrating the skeletal muscle tissues and increasing inflammation. The fungus elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae, a typical characteristic of EUS. The lesion progresses from mild chronic dermatitis to a severe, locally pervasive, necrotizing dermatitis, with severe degeneration of the muscles.



Haematoxylin and Eosin staining demonstrating granulomas indicated by arrows

c. Fungal isolation and identification

This requires patience, time and expertise. The dermal lesions that are moderate, pale and raised are the most suitable for fungal isolation. In order to conduct this procedure, the scales around the periphery of the lesion are removed and the underlying skin is seared with a red-hot spatula to sterilize the surface. Using a sterile scalpel blade and sterile, fine pointed, forceps, the skin is cut horizontally to lift the superficial tissues and expose the underlying muscle. The underlying muscle of approximately 2mm³ is aseptically cut and placed on a Petri dish containing Czapek Dox agar with penicillin G (100 units/ml) and oxolinic acid (100 mg/ml). This is a specialized technique that requires a laboratory with an advanced setting. The inoculated plates are then sealed and incubated at room temperature with daily examination. The emerging hyphal tips are transferred onto fresh plates of Czapek Dox agar until cultures are free of contamination and identified by inducing sporogenesis and PCR.

Level III: Molecular detection and confirmation of EUS. This technique involves molecular confirmation of the genomic DNA of *A. invadans*. The *A. invadans* species specific PCR primers (AIN-VAD2F, TCATTGTGAGTGAAACGGTG, 3' end of the SSU gene; and AINVADITSR1, GGCTAAGGTTTCAGTATGTAG, ITS1) are used. The other two set of primers include (i) FP1 5'-AAG-GCT-TGT-GCT-GAG-CTC-ACA-CTC-3' and FP2 5'-GAT-GGC-TAA-GGT-TTC-AGT-ATG-TAG-3' and (ii) ITS11 5'-GCC-GAA-GTT-TCG-CAA-GAA-AC-3' and ITS23 5'-

CGT-ATA-GAC-ACA-AGC-ACA-CCA-3' can also be used for the detection and confirmation of *A. invadans*. Final confirmation of the *A. invadans* amplified DNA can be done through sequencing.

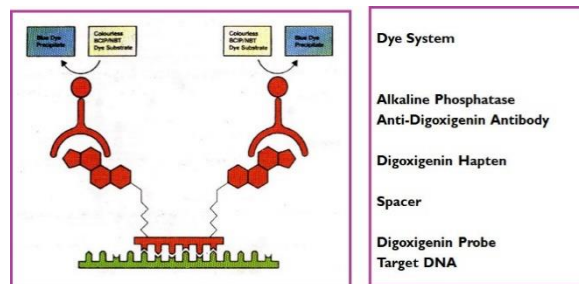
TiLV

Diagnosis of TiLV disease. Examination of histological sections of the primary targeted tissues can provide a presumptive diagnosis of TiLV infection. However, for definitive diagnosis, and to determine the infection status in sub-clinically infected fish, RT-PCR or quantitative reverse transcription polymerase chain reaction (RT-qPCR) testing is recommended. In addition, there are continuous cell culture systems and laboratory infection for the confirmation of TiLV status, therefore the diagnosis can depend on the gross signs, histopathology, RT-PCR-based methods, cell-culture and laboratory infection. With fixed infected tissues, the presence of TiLV can be verified by more specific methods, such as in situ hybridization, or immune-staining assays (e.g. immunohistochemistry or immunofluorescence).

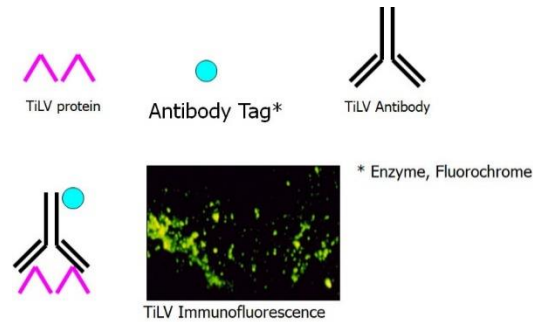
Level I: Gross pathology. The gross signs of TiLV include: skin erosion, hemorrhage at the base of fins and opercula, scale protrusion, abdominal swelling, skin darkening, gill pallor, and ocular alterations. Infected fish also exhibit abnormal behaviour like lethargy, loss of appetite, swimming at the surface, and loss of balance.

Level II: Histopathology. Currently available information suggests syncytial hepatitis to be the most common histopathology feature found in TiLV outbreaks. In the TiLV-infected tilapia, massive cellular necrosis with pyknotic and karyolytic nuclei are found in the hepatocytes; eosinophilic cytoplasmic inclusion bodies are also present in the liver cells.

Level III: In situ hybridization (ISH). ISH is a technique to determine and localize target nucleic acids in fixed tissue sections. Over the years, this method has been improved and greatly increased its sensitivity; it is possible to detect viruses in the cells that no visible signs of infection by histological evaluation. ISH has been applied to reveal the tissue tropism for TiLV. The use of the digoxigenin-labeled probe in conjugation with alkaline phosphatase detection is simple and practical, the tissue sections after color development can be stored for long periods.



Immuno-staining (level III). This is an antigen-detection test that is used either on cell culture (immunocyto-staining) or formalin-fixed tissues (immunohisto-staining). The viral protein (antigen) can be detected through the binding of antibody. For immunohistochemistry (IHC), the virus-specific antibody is tagged with a labeled enzyme (such as alkaline phosphatase or horseradish peroxidase), which will produce intense color precipitates. The stained cells (or tissue sections) can be visualized under the light microscope. For immunofluorescence detection, the virus antibody is tagged with a fluorescence (e.g. FITC) and the stained cells (or tissues) are viewed under a fluorescence microscope.



PCR-based detection (Level III). Several molecular detection methods, including RT-PCR, RT-qPCR, RT loop-mediated isothermal amplification (RT-LAMP) and iPCR protocols have been described for the detection of TiLV. A semi-nested RT-PCR targeting the TiLV genomic segment 3 was developed and shown to have a sensitivity of 7.5 copies of viral sequence. Recently, RT-qPCR methods (both SYBR-Green and TaqMan) targeting the same segment 3 RNA were developed for detection of TiLV with a reported sensitivity of single copies. These methods are listed in the OIE disease card. In addition, a TiLV specific RT-LAMP assay was developed with benefits of reduced analysis time and easy interpretation of results based on the colorimetric change; this method offers a diagnostic tool in resource-limited countries where there is an urgent need for rapid diagnostic assay to guide TiLV control. A commercial TiLV RT-PCR detection assay, based on insulated isothermal polymerase chain reaction (iiPCR, use the TaqMan-based qPCR principle), is available for laboratory and pond-site diagnosis. The hand-held device is called POKKIT™ Micro (manufactured by GeneReach Biotechnology Corp.). The assay can be completed within 45 min and has advantages of being rapid, inexpensive, sensitive, and easy to maintain and operate for non-specialists.

Cell culture and laboratory infection (Level III). The presence of TiLV can be determined by isolating the viruses from the suspect fish and inoculate in continuous cell lines, such as E-11 (derived from snakehead fish, *Ophicephalus striatus*), or primary cell lines. After incubation for approximately 10–14 days at 25°C, cytopathic effect (CPE) can be observed. The purified virions can also be injected intraperitoneally into healthy tilapia, their gross signs and histopathological lesions of injected fish can be observed to determine if the suspect fish, which the inocula were prepared from, are infected by TiLV.

Sample collection and submission. For submitting the samples to a diagnostic laboratory, a detailed case history is important to help to determine the causes of disease. The information should include farm's location, fish species, life stage, sex, gross signs, abnormal behaviour, previous treatments, if any, and the occurrence of morbidity/mortality. Also, senders should specify the purpose of testing for disease monitoring, health screening, surveillance, or certification. Environmental factors include water quality parameters and feeding records will assist to clarify whether handling stress, change of environmental conditions or the presence of infectious agents are causes for concern. These information will also help speed up diagnosis, risk assessment, and husbandry management and treatment recommendations.

Checklist #7: Study design and sampling (N Fejzic)

7	Study design and sampling	Used/described sampling frame 7.a. Described sampling method 7.b Defined sampling units 7.c.Explained consideration regarding sample size Describe tissues/fluids used as sampling material 7.a.1. Describe sample selection process
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Sampling, sampling methods and sample size were critical points in any surveillance because of direct impact on budgetary requirements and consequently on interpretation of surveillance data. According to OIE AAH code (OIE,

2018) the objective of sampling from a population is to select a subset of units from the population that is representative of the population with respect to the object of the study such as the presence or absence of disease. Sampling should be carried out in such a way as to provide the best likelihood that the sample will be representative of the population, within the practical constraints imposed by different environments and production systems. In order to detect the presence of a disease in a population of unknown disease status, sampling methods that optimize the detection of disease can be used. In such cases, care should be taken regarding the inferences made from the results. Random sampling is preferable method, however, in developing countries non random sampling is commonly applied with belonging limitation.

For sampling of wild fish population, a non-random spatial sampling is commonly used. Spatial sampling is similar to random sampling, but instead of selecting individuals from the sampling frame, random locations can be selected from an area. Sampling locations can be determined by measuring the length of the river and randomly selecting numbers along that length. This may be adapted to conform to administrative divisions.

For sampling a farmed fish population, random sampling from the sampling framework (i.e. list of registered/approved/known farms or ponds) is preferred to be used. If data on all registered fish farms exist, first stage of sampling is random selection of appropriate number of farms from a list. However, sampling of farms may not require representativeness (i.e. random selection) in case of sentinel surveillance (as a type of active surveillance) which increases the probability of early detection of a disease given that reliable information on species, time-frame and areas of higher risk are available. At farm level second stage of sampling can refer to selection of ponds/cages or individual animals. This decision relates to type of aquaculture production, aquatic species raised, investigated disease, but foremost to the set objective of surveillance. If objective is to demonstrate disease freedom or early detection, selection of second stage sampling units (ponds, cages, individual animals) begins with likely infected/diseased (increased mortality, moribund or showing signs) and continues with other up to the required sample size. If surveillance is set to establishing disease frequency, random sampling is required. Two-stage sampling can be used even if the number/location of farm is unknown. In this case, a first-stage sampling refers to selection of districts/villages and is followed by a sampling of farms on site. Deciding what in a chain of aquaculture production (district, village, zone, compartment, farm, pond, cage, etc.) is considered sampling unit in any stage of sampling is relevant on case definition (i.e. EUS suspect/infected farm/location) and planned containment/control measures. For farmed fish, this can be a pond (or group of ponds) or a farm if all fish in the same unit share same environmental condition. In such a case, a positive finding of one case of EUS will be interpreted as that the farm is infected regardless how many infected ponds/animals are established on a farm.

Sample size either for the first or second stage of sampling depends on a population size (i.e. number of farms) and farm size (i.e. number of ponds/individual animals), proficiency of diagnostic test used and the surveillance objective from which design prevalence is derived. Design prevalence in the absence of disease specific requirements needs to be set in accordance to the Aquatic Code (OIE, 2018) but in general it represents the minimum expected prevalence of infection in the study population or the prevalence of infection that is practically and reasonably able to be detected by a surveillance system.

If population size, test performance, design prevalence are known (or consciously estimated) and desired estimation error and surveillance sensitivity decided upon (or adopted from international standards), formulas or on line calculators for sample size calculation are openly and easily available via the internet. Since for non-specialist consideration on appropriate sample size might not be so easy to comprehend, for EUS surveillance in participating African countries we used international recommendation (i.e. EC SANCO/6049/2009, OIE Aquatic code) that considering all above sample size determinants.

Example of EUS (if probability sampling is achievable):

Sampling frame	Exist			Don't exist		
Surveillance scenario	Infected	Unknown	Considered free	Infected	Unknown	Considered free
Sampling	Probabilistic			Convenience/purposive/hap hazard		
Design Prevalence	>5 %	50 %	< 2%			
Dg test Sn/Pp			100 %			
Confidence	95 %	95%	95%			
Sample size*						
First stage (epi unit)	73	385	165			
Second stage (within farm)	165	165	165			
Methods	Simple random	Simple random	Simple random			

Sample size, based on defined parameters, calculated using <http://epitools.ausvet.com.au/content.php?page=home>

Example of TiLV (if probability sampling is not achievable):

- Epidemiological Unit: A tilapia farm
- Unit of sampling: A mix of 30 moribund or sick tilapia from ponds at the farm
- Total number of enrolled and participant farms: 40 to 60 tilapia farms, which should be visited twice (total field visits = 80 to 120, per country - at least 1+ve farm, at 2 percent Prev)
- Dynamic of sampling: 1st and 2nd semesters

Checklist #8: Data flow and management (N Fejzic)

8	Data flow and management	<ul style="list-style-type: none"> • Data forms included • Data base (design and management) • 8.a. Compatibility of data throughout the collection/analysis process and transparency • 8.b. Consistency, quality and precision of data
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Data collection and management is the another important element of disease surveillance, since by definition of surveillance (OIE, 2017) it represents data collection activity but inseparable from enabling informed decisions on planning and implementing effective and evidence based health policies. Surveillance systems often incorporated data collection from various sources and using various methodology, but with respect to EUS surveillance, data are collected actively (sampling and filed investigations) and passively (disease suspicion reports and investigations). For each EUS surveillance component, data forms are prepared in advance in form of checklists and questionnaires in order to facilitate and encourage uniform data collection. Questionnaires are developed separately for wild and farmed fish depending on population sampled. It was recommended that after sampling and inspection of fish in a sample, if clinical signs of EUS are observed, two copies of the laboratory questionnaire (form) are completed and one copy sent together with samples to predetermined laboratory and second to competent authority. Data collected in the filed using prescribed data forms, including laboratory results need to be entered into digital data base (i.e. spreadsheet). It is of

key importance to secure traceability of data (farm or population data with laboratory results). In order to secure proper data collection, each country translated the questionnaire/data forms into local language.

Checklist #9: Data analysis and methodology (N Fejzic)

9	Data analysis and methodology	<ul style="list-style-type: none"> • Survey design described • Risk assessment used and described • Methods of data analysis described
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Surveillance activities of sampling and data collection are organized in the format of population based studies/surveys in order to ensure scientific justification, transparency, repeatability and comparisons. The most common epidemiological study design used for surveillance is the cross sectional study. In this type of study, repeated, systemic observation of investigated population are made using uniform sampling protocols with consideration on representativeness of sampling and statistically justified sample size. This enables reliable estimate of a population parameter or characteristic (i.e. prevalence) based on observation made on a sample from that population. Resulting estimates of disease patterns include confidence interval quantifying precision of an estimate. The survey design may involve sampling at several levels (i.e. selection of farms to be investigated following selection of subunits within the farm and/or individual animals within subunits/farms) (OIE, 2018). Other data may be collected for evaluating risk factors for disease occurrence/spread. Using simple data analysis methods (i.e. mapping, stratification of overall results by characteristic such as type and size of farms, location, fish species and age category etc.) important characteristics of disease epidemiology may be recognized and used to prevent spread and support control. Major advantages of this type of study are the ability to objectively estimate disease prevalence, evaluate risk factors, and if surveys are regularly repeated (i.e. annually), to establish disease trends and effectiveness of control measures.

Cross sectional study/survey was selected for the active EUS surveillance in infected and unknown status countries for both wild and farm fish. For wild fish population the survey is designed to provide 95 percent confidence of detecting the presence of EUS, if present in 1 percent of the fish population or more using a diagnostic test with 95 percent sensitivity (Se) and 100 percent specificity (Sp). Consideration about diagnostic test Se and Sp are important since non perfect tests produce false positive and/or false negative results thus affecting desired level of confidence. For farmed fish population the survey is designed to provide 95 percent confidence of detecting the presence of EUS in farms if EUS occurs in 5 percent of the farms or more and if EUS occurs in 2 percent or more within an infected farm using a diagnostic test with 95 percent sensitivity (Se) and 100 percent specificity (Sp). Survey design must include prior estimate/assumption of expected disease parameter which is based on expert opinion, previous studies, expected biology of the agent, information contained in the disease-specific chapter of the OIE Aquatic Manual (OIE, 2018). In addition in all three scenarios (infected, unknown, considered free) passive surveillance should accompany active component. Passive surveillance for the EUS represents reporting of disease suspicion by farmers and local staff/officials in line with case definition.

In disease free countries or if established prevalence by active surveillance (cross sectional study) reveals low prevalence, other surveillance study designs are more appropriate, such as risk-based or targeted surveillance, sentinel surveillance (zoning), syndromic or participatory passive surveillance. Whatever study design is selected it is important that surveillance plan contains description and justification of a selected design and methodology of sampling and data collection. Also expected outputs (measurables) should be defined in advance (in relation to set objectives of surveillance) pertaining to data analysis methodology to be used and thus enabling evaluation. Since surveillance objectives are relevant not only for establishing disease status, but also for choice, timing, scale of interventions and

documenting progress of pathogen or disease reduction, quantitative outputs derived from data analysis are required. Data analysis methodology should be considered in advance (i.e. before sampling and data collection) since it will dictate the type of data to be collected and when, where and how data needs to be obtained.

Example of interpretation of data collected during surveillance:

EUS active surveillance, cross sectional study, two stage sampling design, 95 percent confidence, design prevalence 2–5 percent, farm level prevalence at national level: If 20 percent farms are positive, regardless of within farm prevalence (1 or more cases detected), EUS is presented at 20 percent of farms at national level.

TiLV active surveillance, country considered free, design prevalence 2 percent, cross sectional study, 95 percent confidence, if no cases of disease is detected, we can conclude that TiLV doesn't exist more than 2 percent in country.

Checklist #10: Validation and quality assurance (N Fejzic)

10	Validation and quality assurance	<ul style="list-style-type: none"> • Done by statistical estimation of the level of confidence (Se of surveillance program) • Done by pilot trial • Done by expert/external evaluation (peer-review) • Audit and corrective measures
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Validation is the process that determines the fitness of a surveillance system, which has been properly developed, optimised and standardised for a specific and defined objective. Overestimation or underestimation of parameters of interests were the most common problems in different surveillances. We need validation of surveillance to confirm its scientific value, confidence in system and its compliance with international standards. This step is done throughout the whole process from the design until the actual implementation. The surveillance design and implementation plan might be validated by both data or test validation, pilot studies, external evaluation and peer review evaluation by experts and other relevant project proponents.

Surveillance should incorporate the principles of quality assurance and be subjected to periodic auditing to ensure that all components of the system function and provide verifiable documentation of procedures and basic checks to detect significant deviations of procedures from those documented in the design. Administrative and procedural activities need to be done in order to avoid problems and if problems or mistakes occur, corrective measures can be introduced. These will guarantee good quality implementation of the surveillance plan. For example: national surveillance team (NST) established; training and education of NST on EUS pathogen biology, pathology, diagnostics and surveillance; data collection and a questionnaire described and explained clearly and common understanding achieved; diagnostic laboratory accredited in line with ISO 17025, if possible; trained field and laboratory personnel; a clear standard operating procedures developed and used during implementation, aseptic technique procedures for minimizing contamination from potential areas of sample collection developed and made clear to the sampling teams; sampling teams closely supervised; and a pilot survey will be conducted as a sampling exercise.

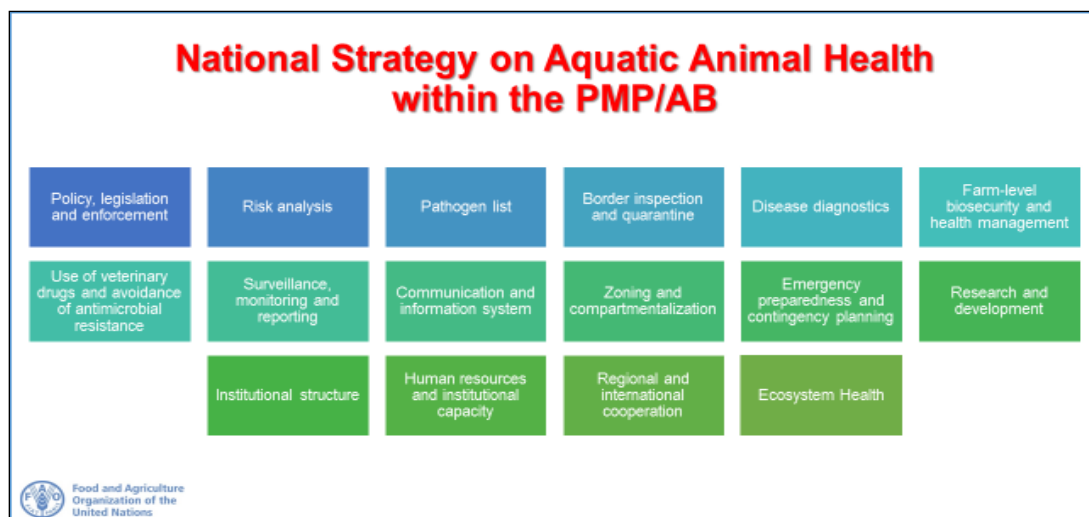
Checklist #11: Human and financial resources and logistics requirements (M Reantaso)

11	Human and financial resources and logistics requirements	Included and described (e.g. personnel, cost of sampling, cost of laboratory tests, analysis of data, etc.)
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Since surveillance is an economic activity, proper planning and costing are essential requirements. Important elements that need to be included in the general planning include the following: (1) list of farmers that will be involved in the surveillance work (from this list, random samples will be taken based on the sampling frame and sampling design); (2) personnel (field surveillance team, laboratory/diagnostics team, data analysis and reporting team); (3) consummables, materials and relevant supplies (field and laboratory) including costs of laboratory tests; (4) training of key personnel; (5) awareness raising of the surveillance activity targeting field and policy level personnel to generate interest and support; (6) all other operational and logistics requirements (transportation, travel expenses, communication, etc.). All of these elements require financial support and estimate costs will be required.

Checklist #12: Putting surveillance in the bigger picture: NSAAH and PMP/AB (M Reantaso)

12	Putting surveillance in the bigger picture (NSAAH and PMP/AB)	Surveillance as an essential component of aquatic animal health/aquatic biosecurity strategies, aquatic animal health protection programmes or disease control plans
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Surveillance is a key element of a national strategy on aquaculture biosecurity and aquatic animal health management and a fundamental of any aquatic animal health protection programme. Surveillance and monitoring programmes are essential for the detection and rapid emergency response to significant disease outbreaks and form the basis for early warning of exotic incursions or newly emerging diseases. They are also increasingly demanded by trading partners to support statements of national disease status and are the basis for disease zonation. Surveillance also provides the building blocks of information necessary to have an accurate picture of the distribution and occurrence of diseases relevant to disease control and international movement of aquatic animals and their products. Surveillance can be passive (reactive and general in nature) or active (proactive and targeted). In both cases, there must be adequate reporting mechanisms so that suspected cases of serious disease are quickly brought to attention of the Competent Authority. Surveillance and monitoring efforts must be supported by adequate diagnostic capability (including appropriately trained


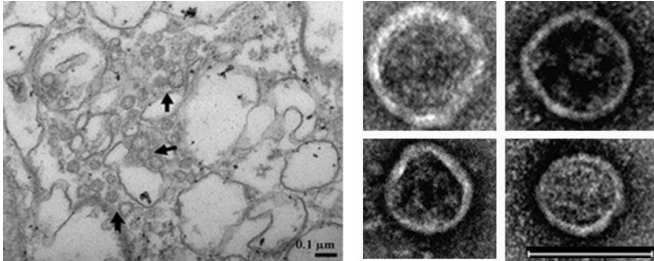
expertise, suitably equipped laboratory and rapid-response field diagnostics, and standardized field and laboratory methods), information system management (i.e. a system to record, collate and analyse data and to report findings), legal support structures, transport and communication networks and linked to national and international (OIE) disease reporting systems (e.g. pathogen list or list of diseases of concern, disease notification and reporting procedures). Surveillance to demonstrate freedom from a specific disease requires a well-designed active surveillance programme that meets the standards outline in the OIE Aquatic Animal Health Code (OIE, 2018).

Annex 1: List of experts and profile


Name	Credentials
Dr Nihad Fejzic	Nihad is an animal health and epidemiology specialist with particular experience in terrestrial and aquatic animal disease surveillance and control, sanitary and phytosanitary measures and management of veterinary services. His area of expertise includes data analysis, system analysis, WTO, OIE and EU sanitary and phytosanitary measures and legislative framework, and international trade of animal products. Dr Fejzic had studied veterinary medicine at University of Sarajevo (1986–1991) and holds a PhD from animal health economics from same University. Currently he is a professor at Veterinary faculty Sarajevo and from May 2012 he is on position of dean of this higher education institution and director of Interdisciplinary One health master study at University of Sarajevo. Dr Fejzic was national coordinator for two FAO projects in BiH (genetic resources and aquatic animal health), and short term expert (trade, SPS, epidemiology animal by products) in several EU projects. He worked as FAO international consultant and expert on epidemiology and aquatic disease surveillance in Asia and Africa focusing in capacity building of aquatic services and surveillance of EUS and TiLV.
Dr Bernard Mudenda Hang'ombe	Bernard is Associate Professor of Microbiology at the University of Zambia, School of Veterinary Medicine. He is responsible for disease diagnosis of animals in aquaculture and terrestrial animals. He has worked with a number of FAO experts in EUS diagnosis and control. He is a beneficiary to a number of many FAO capacity building programmes of aquatic health. Currently he is engaged with fish farmers in the diagnosis and control of diseases especially production diseases such as EUS, Aeromonas, Lactococcus and Streptococcus. He is currently one of the African Union fish health Experts responsible with aquatic health and Biosecurity.
Dr Melba Reantaso	Melba is responsible for Aquaculture Biosecurity at FAO. She worked on EUS between 1985–1988 for the first time while on government service at the Philippine Bureau of Fisheries and Aquatic Resources. She has M.Sc., Ph.D. and post-doctoral qualifications on various aspects of fish health. From 1998–1999 she did her post-graduate studies on EUS at the Nippon Veterinary and Animal Science University as a fellow of the Japan Society for the Promotion of Science (JSPS) under the supervision of Prof. Kishio Hatai, the scientist who first discovered and described EUS as of fungal aetiology. She led the international task force that investigated for the first time the occurrence of EUS in Botswana and two sub-regional projects on EUS in the African region. She initiated efforts in capacity building on diagnostics and surveillance for EUS and TiLV (plus risk analysis for the latter) with ongoing and pipeline projects in Africa, Asia and South America.
Dr Mwansa Songe	Mwansa is head of Aquatic Animal Health in the Department of Veterinary Services, Ministry of Fisheries and Livestock. She has a PhD in Aquatic Medicine from The Norwegian University of Life Sciences. She first worked on EUS following the very early outbreak of the disease in Zambia and other countries sharing the Zambezi River. She did her post graduate studies from 2007–2009, focusing on the pathological changes in fish species infected with EUS in the Zambezi River basin, with financial support from the then Ministry of Science and Technology. Under the supervision of Prof. Bernard Hang'ombe, with technical support from experts at FAO and the Aquatic Animal Health Research Institute in Thailand, she investigated the pathogenicity and infectivity of <i>Aphanomyces invadans</i> , the causative agent of EUS. She is currently involved in fish disease surveillance focusing on screening for EUS and TiLV, supported by WorldFish.

<p>Dr Win Surachetpong</p>	<p>Win is an associate professor at Department of Veterinary Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Thailand. He has worked on tilapia lake virus (TiLV) in Thailand since 2015 when the virus was first identified in Asia. Win finished his Doctor of Veterinary Medicine from Chulalongkorn University in 2000. He did his Master of Science in Pathobiology at University of Arizona under the supervision of Professor Donald Lightner on shrimp pathology. Then, he received a PhD in immunology from University of California, Davis. Currently, Win research emphasizes on TiLV in the area of epidemiology, diagnostic methods, host-pathogen interaction, risk factor analysis of the disease, biosecurity and vaccine development. Win has been participated as a speaker and expert on TiLV for international conference, workshop and training during the last few years.</p>
<p>Dr Kathy (Feng-Jyu) Tang-Nelson</p>	<p>Kathy is a Resource Expert in aquatic animal health for the FAO. She was a faculty member at University of Arizona from 1997–2017 and is currently a research affiliate of the Yellow Sea Fisheries Research Institute in Qingdao, China. During her time at the University of Arizona, and after, she has authored numerous publications on bacterial, viral and microsporidian diseases of aquaculture species. She has developed molecular diagnostic methods, such as quantitative PCR and in situ hybridization, for pathogens of aquatic animals. Her recent work with tilapia lake virus (TiLV) includes preparation of a disease strategy manual that provides key information, for national policymakers, producers, and other stakeholders, relevant to the development of TiLV contingency plans. She also has provided training in PCR-based diagnostic methods to researchers in SE Asia and Africa and advice regarding the equipment, supplies and procedures required for setting up a competent diagnostic laboratory.</p>

Annex 2: Tilapia lake virus (TiLV) disease (K Tang-Nelson/W Surachetpong)

BACKGROUND INFORMATION	
Causative agent	<p>Tilapia lake virus (TiLV)* disease was recognized in Israel starting in 2009, where it caused significant mortality in wild and cultured tilapia (Eyngor <i>et al.</i>, 2014; Bacharach <i>et al.</i>, 2016) (Fig.1). In 2011, through viral isolation and laboratory infections, the causative agent was identified as an enveloped, negative-sense, RNA virus. Transmission electron microscope (TEM) show round enveloped virions with diameters of 70–110 nm (Fig.2) (Eyngor <i>et al.</i>, 2014; Tattiyapong <i>et al.</i>, 2017). The virion has a capsid containing several (up to 7) electron-dense aggregates within an electron-dense core (del-Pozo <i>et al.</i>, 2017).</p>
	 <p>Fig.1. The occurrence of mass mortality due to TiLV in hybrid tilapia (<i>Oreochromis niloticus</i> x <i>O. aureus</i>) cultured in Israel. (Photo courtesy of Dr. Bacharach).</p>
	 <p>Fig.2. Transmission electron micrographs (TEM) of TiLV showing round-shaped virions. (A) Within the cytoplasmic area of a brain cell; (B) purified virion, scale bar=0.1 μm. (Photos courtesy of Dr. Surachetpong and Dr. Bacharach).</p>
	<p>By genomic sequence analysis, TiLV has recently been placed as a new species, <i>Tilapia tilapinevirus</i>, under a new genus, <i>Tilapinevirus</i>, in a currently unassigned family (Adams <i>et al.</i>, 2017). This virus contains 10 segments of RNA, each of which contains an open reading frame (ORF) flanked by non-coding regions (NCRs) at 3' and 5' ends. The size of each segment ranges from 456 (segment 10) to 1641 (segment 1) nucleotides and the total genome size is 10,323 nucleotides (Bacharach <i>et al.</i>, 2016). The ORF (519 amino acid) of segment 1 encodes a putative RNA-dependent RNA polymerase (RdRp) containing a conserved domain of the influenza C virus RdRp subunit PB1. However, the amino acid sequence similarity of this putative RNA polymerase is only 17 percent to that of the influenza C virus. The ORFs from 9 other segments show no similarity to known viruses. All of the TiLV genomic segments have highly conserved sequences at the 3' and 5' termini, both with 13 nucleotides. The inverted, partially, complementary termini may form a duplex structure (referred as a panhandle), which can function as a promoter for viral transcription and replication as seen in influenza viruses (Hu <i>et al.</i>, 1987).</p>
	<p>By analyzing the protein contents of the TiLV virions with liquid chromatography-tandem mass spectrometry (LC MS/MS), 9 proteins predicted from ORFs of segments 2 to 10 were identified. No protein of encoded from ORF of segment 1 (putative RdRp) was found in the purified virions (Bacharach <i>et al.</i>, 2016).</p>
	<p>TiLV is not yet listed by the OIE as a notifiable disease.</p>

	*TiLV disease was also named as syncytial hepatitis of tilapia (SHT) By Ferguson <i>et al.</i> (2014) and del-Pozo <i>et al.</i> (2017).
Host Range	<p>Farmed and wild tilapia are infected by TiLV, including hybrid tilapia (<i>Oreochromis niloticus</i> x <i>O. aureus</i> hybrids), Nile tilapia (<i>O. niloticus</i>), Gray tilapia (<i>Oreochromis niloticus</i> x <i>O. aureus</i>) and red tilapia (<i>Oreochromis</i> sp.) (Eyngor <i>et al.</i>, 2014; Ferguson <i>et al.</i>, 2014; Fathi <i>et al.</i>, 2017; Dong <i>et al.</i>, 2017a; Mugimba <i>et al.</i>, 2019; Surachetpong <i>et al.</i>, 2017).</p> <p>TiLV has also been identified from various species of wild tilapines including <i>Sarotherodon galilaeus</i>, <i>Tilapia zilli</i>, <i>Oreochromis aureus</i> and <i>Tristramellasisimonis intermedia</i> in the Sea of Galilee, Israel (Eyngor <i>et al.</i>, 2014).</p> <p>Other fish species have been shown to susceptible to TiLV infection including giant gourami (<i>Osphronemus goramy</i>) while most warm water fishes are resistant to TiLV infection (Jaemwimol <i>et al.</i>, 2018). Besides, the viral RNA was detected in wild river barb (<i>Barbonymus schwanenfeldii</i>) in the man-made lake that has diseased tilapia (Abdullah <i>et al.</i>, 2018).</p>
Affected population (wild, farmed, both), and life stages; mortality rates	<p>TiLV disease can occur at any stage of the fish including fertilized eggs, yolk sac larvae, fry, fingerlings, juveniles and adults (Eyngor <i>et al.</i>, 2014; Ferguson <i>et al.</i>, 2014; Dong <i>et al.</i>, 2017a, 2017b; Surachetpong <i>et al.</i>, 2017; Yamkasem <i>et al.</i>, 2019).</p> <p>During the initial outbreak of TiLV disease, mortality can reach 90 percent. However, once the initial mortality ceases, no further mortalities occur in the infected populations. This suggests that the surviving tilapia possess protective immunity against this virus. This has also been demonstrated in laboratory infection studies where fish that survived one intraperitoneal injection of TiLV were completely immune to the disease upon a second injection 3–4 weeks later (Eyngor <i>et al.</i>, 2014).</p>
Environmental information	<p>The disease outbreaks were associated with the warm season when water temperature reached 22°C–32°C in Israel during the months of May to October (Eyngor <i>et al.</i>, 2014). The disease was also observed during the months of June–October in Egypt when water temperature was >25°C. When this was observed, the event caused by TiLV was referred to as “summer mortality” (Fathi <i>et al.</i>, 2017).</p> <p>It has been observed that higher percentage of TiLV disease occurred in farms with larger size fish at high stocking density. It was also observed in tilapia-mullet polyculture, where the mullet appeared resistant to TiLV disease (Fathi <i>et al.</i>, 2017).</p>
Economic importance	<p>The culture of tilapia, the common name for taxonomically related cichlid fishes primarily in the genera <i>Tilapia</i>, <i>Oreochromis</i>, and <i>Sarotheradon</i>, is a major component of global aquaculture and tilapias are very important species in international food security programs. Tilapia provides inexpensive dietary protein in the developing countries, thus the emergence of the TiLV, which has been shown to cause substantial mortality (80–90 percent) in farmed populations of tilapia, poses a serious risk to aquaculture producers and to global food security. Global production of tilapia was estimated at 2.5 million tons with a value of over USD 7.5 billion (FAO, 2014).</p> <p>In Egypt, TiLV disease caused production losses of 98,000 tons at a value of USD 100 million during 2015 (Fathi <i>et al.</i>, 2017). Egypt is the third largest tilapia producing country in the world.</p>
Geographic Distribution	<p>TiLV was first recognized in Israel in 2009 (Eyngor <i>et al.</i>, 2014; Bacharach <i>et al.</i>, 2016). Subsequently, the disease was reported in several other countries: Ecuador (started in 2011) (Ferguson <i>et al.</i>, 2014; del-Pozo <i>et al.</i>, 2017; Kembou Tsofack <i>et al.</i>, 2017), Colombia, Thailand (2015) (Dong <i>et al.</i>, 2017a, 2017b; Surachetpong <i>et al.</i>, 2017), Egypt (2016) (Fathi <i>et al.</i>, 2017; Nicholson <i>et al.</i>, 2017), Tanzania (2015), Uganda (2016) (Mugimba <i>et al.</i>, 2018), India (2016) (Behera <i>et al.</i>, 2018), Taiwan (2017; OIE WAHIS Interface, 2017a), the Philippines (2017; OIE WAHIS Interface, 2017b), Malaysia (2017) (Amal <i>et al.</i>, 2018), Peru (OIE WAHIS Interface, 2018), and most recently Mexico and USA (OIE WAHIS interface, 2019). However, a lack of comprehensive investigation of mortality-associated incidents in tilapia could mean that the geographic distribution of TiLV may be wider than currently known. For</p>

	<p>example, reports of substantial mortality in tilapia in Ghana and Zambia in 2016 were not well investigated to determine if TiLV was the causative agent.</p> <p>Dong <i>et al.</i> (2017b) used RT-PCR to screen tilapia samples, archived and fresh, from four hatcheries in Thailand for the presence of TiLV. A majority of these samples, which were collected during the period of 2012–2017, tested positive for TiLV. Since many countries import tilapia fry and fingerlings from Thailand, it is possible, although no specific instances have been documented, that TiLV could have been spread to other countries through exports of fry or fingerlings.</p>
Epidemiological data	<p>There appears to be a number of TiLV genotypes that are associated with a range of fish species and related pathologies (Tattiyapong <i>et al.</i>, 2017). The TiLV isolates from around the world have over 90 percent identities in nucleotide sequence (Jansen and Mohan, 2017). Partial genomic sequences from Ecuador, Thailand and India showed 96–100 percent identities (nucleotide sequence) to the reference strain of Israel (segment 1, Genbank no. KU751814) (del-Pozo <i>et al.</i>, 2017; Dong <i>et al.</i>, 2017a; Behera <i>et al.</i>, 2018). Comparison of the nucleotide sequence of segment 3 of the Egypt and Thailand isolates showed 93 and 98 percent identities, respectively, with the Israel isolate (Dong <i>et al.</i>, 2017a; Fathi <i>et al.</i>, 2017). The nucleotide sequences (segment 2) of Tanzania and Uganda isolated from Lake Victoria had >99.8 percent identities, and they clustered with strains from Israel and Thailand by a phylogenetic analysis, suggesting that TiLV in Thailand, Israel and Lake Victoria may have a common origin (Mugimba <i>et al.</i>, 2018).</p>
DIAGNOSIS	
Level I: Gross signs and behaviour	<p>TiLV disease gross signs include: skin erosion, hemorrhage at the base of fins and opercula (Fig. 3A and 3B), scale protrusion and loss (Fig. 3B), abdominal swelling due to the presence of ascites, skin darkening, gill pallor, and ocular alterations such as exophthalmia (“pop-eye”), shrinkage of the eyeball (phthisis bulbi) in severe infection (Fig. 3C) and opacity of the lens. Fish also exhibit abnormal behaviour like lethargy, loss of appetite, swimming at the surface, and loss of balance (Eyngor <i>et al.</i>, 2014; Ferguson <i>et al.</i>, 2014; Dong <i>et al.</i>, 2017a; Surachetpong <i>et al.</i>, 2017; Tattiyapong <i>et al.</i>, 2017).</p> <div data-bbox="418 1121 1409 1346" style="text-align: center;">  </div> <div data-bbox="418 1356 1463 1560" style="border: 1px solid black; padding: 5px;"> <p>Fig.3. Gross signs of TiLV disease in clinical samples. (A) diseased red tilapia showed hemorrhage (black arrows); (B) diseased Nile tilapia showed skin erosion, hemorrhage on various parts of body, loss of scales, abdominal swelling, and exophthalmos; (C) diseased wild tilapia (<i>Sarotherodon galilaeus</i>) showed shrinkage of the eye and loss of ocular functioning (phthisis bulbi). (Photos courtesy of Dr. Surachetpong and Dr. Bacharach).</p> </div>
Level II: Histopathology	<p>For histology, moribund fish are euthanized by eugenol solution, fixed in 10 percent neutral buffered formalin, processed for tissue sectioning, followed by hematoxylin and eosin (H&E) staining using the standard procedures.</p> <p>Histological examinations show lesions in the brain, liver, spleen, eye and kidney.</p> <p>The lesions of the brain include edema, focal hemorrhages in the leptomeninges, congestion of blood vessels (Fig. 4A), and congestion in both the white and gray matter; perivascular cuffs of lymphocytes</p>

are detected (Fig. 4B). Some neurons within the telencephalon, particularly in the optic lobes, display various levels of degeneration (Eyngor *et al.*, 2014; Tattiyapong *et al.*, 2017).

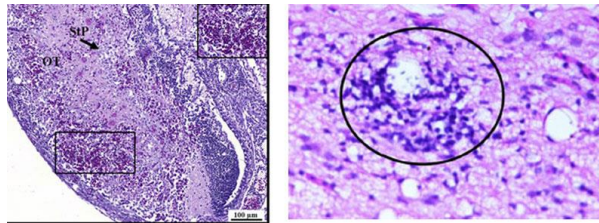


Fig.4. Histopathology of brain in TiLV-infected tilapia. (A) Multifocal hemorrhages with severe blood congestion in the brain, (B) perivascular cuffs of lymphocytes (encircled). (Photos courtesy of Dr. Surachetpong and Dr. Bacharach).

In the liver, there are syncytial cell formation and massive hepatocellular necrosis with pyknotic and karyolytic nuclei. Eosinophilic cytoplasmic inclusion bodies are also present in the liver cells (Fig. 5) and syncytial formation of the liver cells with multiple nuclei (Fig. 6). Perivenular lymphocytes are also observed.

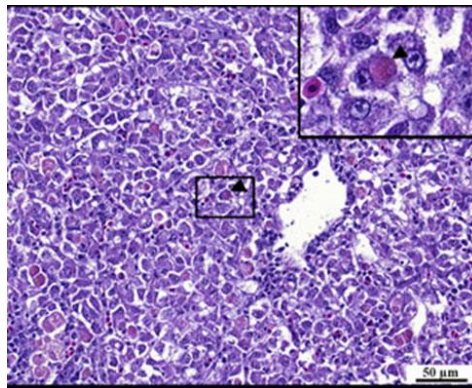


Fig.5. Histopathology of liver in TiLV-infected fish. Extensive hepatocellular necrosis with eosinophilic intracytoplasmic inclusion body in liver cells (higher magnification). (Photo courtesy of Dr. Surachetpong).

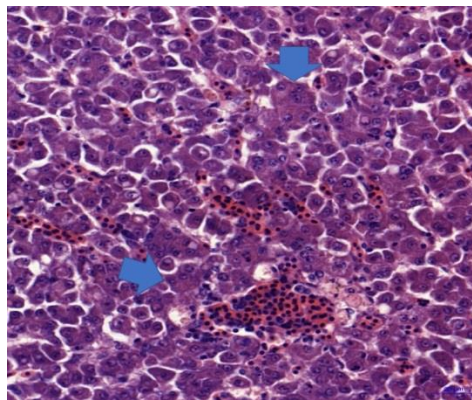


Fig.6. Histopathology of liver in TiLV-infected fish. Syncytial cell formations with multiple nuclei (arrow heads). (Photo courtesy of Dr. Surachetpong).

In the spleen, the size and number of melanomacrophage center (MMC, known as macrophage aggregates) are increased and eosinophilic cytoplasmic inclusion bodies are present (Fig. 7).

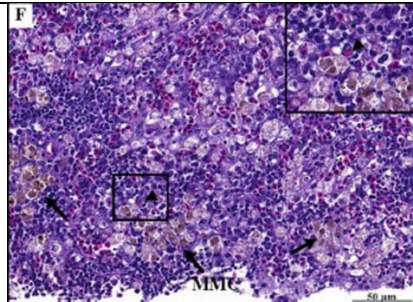


Fig.7. Histopathology of spleen in TiLV-infected tilapia. Increased melanomacrophage center (MMC) (arrows) with eosinophilic intracytoplasmic inclusion body (arrow head, higher magnification). (Photo courtesy of Dr. Surachetpong).

The ocular lesions included ruptured lenticular capsule and cataractous changes characterized by the appearance of eosinophilic homogenous spherical structures (known as morgagnian globules) (Fig. 8).

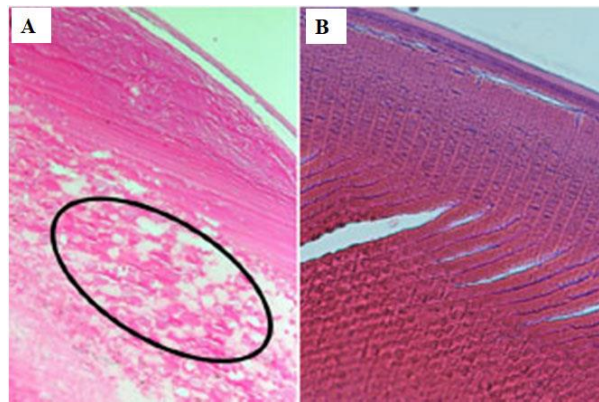


Fig.8. Histopathology of lens in TiLV-infected tilapia. (A) Cataractous changes characterized by formation of eosinophilic spherical structures accompanied by degeneration of crystalline fibers (encircled); (B) control lens from healthy fish. (Photo courtesy of Dr. Bacharach).

In the kidney, multiple necrotic foci were observed (Fig. 9).

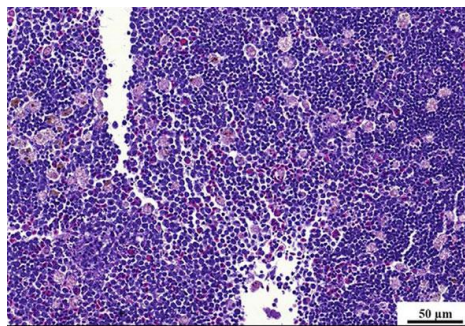


Fig.9. Histopathology of kidney in TiLV-infected tilapia fish showing multifocal area of necrosis and inflammatory cells infiltration. (Photo courtesy of Dr. Surachetpong).

Level III: Other Relevant diagnostic techniques

Cell culture. The presence of TiLV can be determined by isolating the viruses from the suspect fish using tissue homogenates inoculated in continuous cell lines such as E-11 (derived from snakehead), CFF (from *Pristolepis fasciatus*), OmB (from *Oreochromis mossambicus*) and TmB (from *Tilapia mossambica*) incubated under optimal temperature for TiLV replication at 25°C. After inoculation with virus-suspect tissue homogenate, the cultured cells are observed daily for the appearance of cytopathic effect (CPE). Infected cells display shrinkage, aggregation, rounding up and detach from the culture surface. TiLV-positive samples will induce CPE in the cell lines after 4–12 days (Eyngor *et al.*, 2014; Kembou Tsofack *et al.*, 2017; Tattiyapong *et al.* 2017; Behera *et al.*, 2018).

TiLV can also multiply in the primary tilapia brain cells incubated at optimal temperature of 25°C. The CPE is characterized by conversion of the typical elongated cells into swollen, rounded, and granulated

	<p>cells, which are clearly observed at 10–20 days post-inoculation, leading to large monolayer detachment (days 14–19), but without plaque formation.</p> <p>The tissue homogenate can be prepared by homogenizing minced TiLV-infected tissues (such as brain, liver) into Hank's balanced salt solution (w/v: 1/10), and clarified at 3,000 x g for 10 min at 4°C. The supernatant can be filtered through a 0.22 µm filter and then inoculated into confluent cells.</p> <p>Bioassay. Naive tilapia are maintained in a control facility at 28°C with aeration and fed with a commercial diet daily. The TiLV inoculum can be obtained from filtered (0.22 µm) tissue homogenate or could come from the cell culture supernatant, which may be kept frozen at -80°C before use. The optimal dosage (e.g. injecting 1 x 10⁶ TCID₅₀ of cell culture supernatant to each fish) can be injected intraperitoneally into each healthy fish. Recently, a study showed that the intragastric challenge could produce clinical disease under laboratory condition, suggesting that the virus could enter fish via gastrointestinal route (Pierezan <i>et al.</i>, 2019). The injected fish will be monitored for the appearance of gross signs and associated mortalities for two weeks. Any dead or moribund fish will be removed and examined for the presence of gross lesions, then the target tissues (such as brain, liver) will be sampled for TiLV by RT-PCR detection.</p> <p>RT-PCR. RT-PCR and RT-qPCR have been used for the diagnosis of TiLV. Details on how to perform these tests can be found in the original publications and in the OIE technical disease card (OIE, 2017). The tissues to be analysed include liver, brain, eye, anterior kidney and spleen. A TiLV RT-PCR method was developed targeting the segment 1 (Eyngor <i>et al.</i>, 2014). Later, a nested RT-PCR assay targeting the segment 3, with an improved sensitivity, enable the detection limit down to single copies (7 copies) of TiLV (Kembou Tsofack <i>et al.</i>, 2017). RT-qPCR methods has been developed with a detection limit of 2 viral copies, a method that is 100 times more sensitive than the conventional RT-PCR method (Tattiyapong <i>et al.</i>, 2018). A Taqman quantitative PCR protocol was developed for TiLV with specific probe for higher specificity (Waiyimitra <i>et al.</i>, 2019). Additionally, a RT-Loop Mediated Isothermal Amplification (RT-LAMP) method based on the colorimetric change could be applied for TiLV detection in the field (Phusantisampan <i>et al.</i>, 2019)</p> <p>A non-invasive sampling TiLV from fish mucus was demonstrated, this mucus can be used for the diagnosis of TiLV by RT-PCR and cell-culture analyses (Liamnimitr <i>et al.</i>, 2018)</p> <p>Commercial PCR and qPCR kits for detection of TiLV are also available, for example, IQ REAL TiLV Quantitative System; and the IQ Plus TiLV kit for pond-side diagnosis.</p> <p>In situ hybridization (ISH). By ISH, several tissues were found positive for TiLV, including liver, kidney, brain, gills, spleen, reproductive organs, and connective tissue in the muscle, strongest signal was observed in the liver (Bacharach <i>et al.</i>, 2016; Dong <i>et al.</i>, 2017a; Jaemwimol <i>et al.</i>, 2018; Yamkasem <i>et al.</i>, 2019).</p>
MODES OF TRANSMISSION	
Vectors/Carriers	Not reported.
Disease transmission to animals	TiLV can be transmitted horizontally via contaminated water (a waterborne disease) or contaminated sediments in the pond bottom. There is no evidence of vertical transmission. TiLV was detected in samples of fertilized eggs and yolk-sac larvae (Dong <i>et al.</i> , 2016b). Recent study demonstrated that TiLV could be detected in the reproductive organs of male and female fish suggesting the potential transmit via vertical transmission from brood stock to progenies (Yamkasem <i>et al.</i> , 2019)

Disease spread between groups of animals	The disease can be transmitted from infected fish with small abrasions in the skin, or injuries in scales, which are in a close contact with non-diseased fish. In addition, live viruses can be detected in the mucus of infected fish, the disease can be transmitted through cohabitation proven by laboratory bioassays (Liamnimitr <i>et al.</i> , 2018).
PUBLIC HEALTH	
Food safety Zoonosis	The TiLV is not a human pathogen. There is a study demonstrating that there is relatively low risk of potential transmission of the virus via frozen tilapia fillet (Thamatorn <i>et al.</i> , 2019)
PREVENTION	
	<p>Improvement in environmental conditions, not to stock the fish during the hot season and reduction of stocking density are the usual preventive measures for TiLV disease in farmed tilapia. Removal of the moribund and dead fish can also decrease the risk of disease outbreaks. Most common disinfectants in aquaculture facility including hydrogen peroxide, formalin, sodium hypochlorite, povidone-iodine complex, chlorine, and Virkon® have been shown to effectively inhibit virus under tilapia aquaculture practice in most temperate countries (Jaemwimol <i>et al.</i>, 2019; Soto <i>et al.</i>, 2019)</p> <p>The development of vaccines is ongoing. The tilapia farming industry also relies on biosecurity measures to prevent the introduction of TiLV to reduce the spread of this disease. Among these has been the use of sensitive diagnostic tools, such as RT-qPCR and/or cell culture method for screening broodstock and/or fingerlings and destroying TiLV-positive animals.</p>
CONTROL MEASURES	
	<p>Good aquaculture practices (GAPs) are the best safeguards against TiLV, these include, among others: (1) screening imported stocks via accurate diagnostics; (2) stocking farms with fries from verified, clean hatcheries; (3) disinfection of facilities and ponds after outbreaks; (4) restricting the movement of live infected tilapia from farms and open waters; (5) reducing stress on farmed populations by maintaining good water quality and aeration; (6) implementing rigorous biosecurity measures to minimize spread via equipment, vehicles, or personnel.</p> <p>Currently, there are no methods other than GAPs that have been shown to be effective in limiting the impact of an outbreak in particular farms. However, fish that survive TiLV disease outbreaks become resistant to subsequent outbreaks (Eyngor <i>et al.</i> 2014), so breeding for resistance may offer the long-term prospects for managing the disease. In addition, commercial fish feeds containing immunostimulants (such as β-glucans), prebiotics (such as mannoooligosaccharides), probiotics, or combinations of these are available. However, their effectiveness against TiLV is not fully known.</p> <p>There are no vaccines available for TiLV although work toward their development is underway in Israel and Thailand.</p>

Annex 3: Draft Programme

Date	Programme
13 Oct (Sun)	Arrival of participants to Lusaka
14 Oct (Mon)	Day 1: Opening and Sessions 1, 2 and 3
08:00	Pick-up from hotel to course venue
08:30-09:00	Registration
09:00-09:45	Opening session Opening Prayer Introduction of the participants Opening Remarks (ZAEDP Coordinator) Welcome Remarks (Dean, School of Veterinary Medicine, UNZA) Welcome Remarks (FAO Representative to Zambia) Official Opening (Permanent Secretary, Ministry of Fisheries and Livestock)
09:45-10:00	Group photo
10:00-10:30	Coffee
	Session 1: Introduction to the Training/Workshop: FAO 12-point surveillance checklist (for non-specialists and for developing countries)
10:30-11:00	Introduction to the project and Training/Workshop Four P's (Melba)
11:00-12:30	Session 2: Introduction to Disease diagnostics and surveillance, EUS and TiLV
	<ul style="list-style-type: none"> • Factors in disease development (Snieszko circle): lecture (Melba) • Diagnostics (Levels I, II, III): lecture (Melba) • What is epidemiology? What is surveillance?: lecture (Nihad) • Importance of surveillance in aquatic animal health management: lecture (Nihad)
12:30-13:30	Lunch
13:30-15:00	Introduction to EUS: lecture (Bernard/Mwansa)
	Introduction to TiLV: lecture (Win)
15:00-15:20	Coffee
15:20-17:30	Session 3: FAO 12-point diseases surveillance checklist
15:20-15:40	Introduction to the 12-point checklist: Active surveillance for EUS and TiLV: lecture (Melba)
15:40-16:00	Checklist 1: Hazard/disease scenario setting: lecture (Melba)
	Checklist 2: Defining data collection objective: lecture (Melba)
16:00-16:30	Working Group (WG) Exercise 1 and 2 and presentations (Melba)
16:30-17:00	Checklist 3: Definition of population: lecture (Nihad)
	Checklist 4: Clustering of disease: lecture and discussions (Nihad; Mwansa/Bernard for EUS; Win/Kathy for TiLV)
	Checklist 5: Case/outbreak definition: lecture and discussions (Nihad: Mwansa/Bernard for EUS; Win/Kathy for TiLV)
17:00-17:30	Working Group (WG) Exercise Checklist 3, 4 and 5 and presentations (Nihad)
15 Oct (Tues)	Day 2: Checklist 6: Diagnostics/Testing
07:00	Pick-up from hotel to course venue
07:30-08:00	Wrap up of Day 1 and Tasks for Day 2
08:00-17:30	Checklist 6: Diagnostics/Testing
08:00-09:00	EUS diagnostics: Level I, II, III (lecture): Mwansa/Bernard
09:00-10:00	TiLV diagnostics: Level I, II, III (lecture): Kathy/Win
10:00-10:20	Coffee
10:20-12:30	Laboratory work <ul style="list-style-type: none"> • EUS diagnostics (laboratory): Level I, II, III demo, forms

	<ul style="list-style-type: none"> • TiLV diagnostics (laboratory): Level I, II, III demo, forms
12:30-13:30	Lunch
13:30-17:30	Laboratory work (continued): practice
15:00-15:20	Coffee
16 Oct (Wed)	Day 3: Checklists 7, 8, 9, 10, 11
07:00	Pick-up from hotel to course venue
07:30-08:00	Wrap up of Day 2 and Tasks for Day 3
08:00-10:00	Checklist 7: Study design and sampling (Nihad)
	Working Group Exercise Checklist 7 (Nihad)
10:00-10:20	Coffee
10:20-12:30	Checklist 8: Data flow and management
	Lecture, forms (data sheet) and flowchart
	Checklist 9: Data analysis and methodology
	Lecture, forms (data sheet) and flowchart
	Checklist 10: Validation and quality assurance (Nihad)
	Lecture, forms (data sheet)
	Checklist 11: Human and financial resources and logistics requirements (Melba)
	Lecture, forms
12:30-13:30	Lunch
13:30-18:00	Working Group (WG) Exercise Checklist 7, 8, 9, 10, 11 and presentations (Nihad and Melba)
17 Oct (Thurs)	Day 4: Checklist 12 and the Way Forward
07:00	Pick-up from hotel to course venue
07:30-08:00	Wrap up of Day 2 and Tasks for Day 3
08:00-08:30	Checklist 12: Putting surveillance in the bigger picture (NSAAH and PMP/AB)
08:30-12:00	Final review of active surveillance plan for EUS and TiLV (Melba)
	The Way forward (Melba)
	Closing and distribution of certificates (Francis/Mwansa)
12:00-13:00	Lunch