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REVIEW

REVIEWS IN Aquaculture

From the basics to emerging diagnostic technologies: What is on the horizon for tilapia disease diagnostics?

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Abstract

Tilapia is an affordable protein source farmed in over 140 countries with the majority of production in low- and middle-income countries. Intensification of tilapia farming has exacerbated losses caused by emerging and re-emerging infectious diseases. Disease diagnostics play a crucial role in biosecurity and health management to mitigate disease loss and improve animal welfare in aquaculture. Three continuous levels of diagnostics (I, II and III) for aquatic species have been proposed by Food and Agriculture Organization of the United Nations (FAO) and the Network of Aquaculture Centers in Asia and the Pacific (NACA) to promote the integration of basic and advanced methods to achieve accurate and meaningful interpretation of diagnostic results. However, the recent proliferation of cutting-edge molecular methods applied

Abbreviations: AI, artificial intelligence; ARG, antimicrobial resistant gene; AST, antimicrobial susceptibility test; AuNP, gold nanoparticles; Cas, CRISPR-associated protein; CPA, cross-priming amplification; CRISPR, clustered regularly interspaced short palindromic repeats; Ct, cycle threshold; dPCR, digital polymerase chain reaction; eDNA, environmental deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; eRNA, environmental ribonucleic acid; HAD, helicase-dependent amplification; IAM, isothermal amplification method; iiPCR, insulated isothermal PCR; IR, infra-red; ISH, in situ hybridization; LAMP, loop-mediated isothermal amplification; LFIA, lateral flow immunoassay; LoD, limit detection; MLST, multilocus sequence typing; mNGS, metagenomic next generation sequencing; NASBA, nucleic acid sequence-based amplification; NGS, next generation sequencing; ONT, Oxford Nanopore Technologies; PCR, polymerase chain reaction; POCT, point-of-care testing; qPCR, quantitative real-time polymerase chain reaction; RCA, rolling circle amplification; RPA, recombinase polymerase amplification; RT-LAMP, reverse transcriptase loop-mediated isothermal amplification; RT-PCR, reverse transcriptase-polymerase chain reaction; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; RT-RPA, reverse transcriptase recombinase polymerase amplification; SMRT, single molecule real-time; TEM, transmission electron microscopy; TGS, third generation sequencing; WGS, whole genome sequencing; WOAH, the World Organisation for Animal Health.

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in the diagnosis of diseases of aquacultured animals has shifted the focus of researchers and users away from basic approaches and toward molecular diagnostics, despite the fact that many diseases can be rapidly diagnosed using inexpensive, simple microscopic examination and that most emerging diseases in aquaculture were discovered by histopathology. This review, therefore, revisits and highlights the importance of the three levels of diagnostics for diseases of tilapia, particularly the frequently overlooked basic procedures (e.g., case history records, gross pathology, presumptive diagnostic methods and histopathology). The review also covers current and emerging molecular diagnostic technologies for tilapia pathogens including polymerase chain reaction methods (conventional, quantitative, digital), isothermal amplification methods Loop-mediated Isothermal Amplification (LAMP), recombinase polymerase amplification (RPA), clustered regularly interspaced short palindromic repeats (CRISPR)-based detection, lateral flow immunoassays, as well as discussing what is on the horizon for tilapia disease diagnostics (next generation sequencing, artificial intelligence, environmental Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA) and point-of-care testing) providing a future vision for transferring these technologies to farmers and stakeholders for a sustainable aquatic food system transformation.

KEYWORDS
disease, basic diagnostics, emerging technologies, tilapia

1 | OVERVIEW OF TILAPIA AQUACULTURE, DISEASES AND IMPORTANCE OF DIAGNOSTICS

Aquatic foods, both farmed and caught, distributed through various supply chains, have significantly contributed to the improvement and diversification of diets, as well as the promotion of nutritional well-being for many people.[1] Recognition of the critical role of aquatic foods in nourishing nations and transforming food systems is increasing with the recent tabling of a discussion paper by the United Nations (UN) on the role of aquatic foods for nutrition gaining global attention.[2] In addition to underpinning local nutritional needs and livelihoods for tens of millions of people, aquatic commodities are some of the most traded food products in the world. The value of global fish exports increased from USD 7.8 billion in 1976 to USD 164 billion in 2018.[3] Producing aquatic foods that are safe, healthy, accessible and affordable is the need of the hour to meet the nutritional needs of millions of people. This is where the farming of carps, tilapias and catfish assumes significance and presently supply 35.84% of world aquaculture production with a value of 83 billion dollars.[3,4]

Tilapia, by virtue of their overall resilience, have been species of choice for farming in a diverse range of farming systems, from simple backyard/homestead ponds to highly intensive raceways. Today, tilapia is the second most commercially important finfish group after carps, farmed in over 140 countries.[3,5–7] In 2018, global tilapia production by volume was estimated at 6.5 million metric tonnes (MMT) with the top four producers being China (1.78 MMT), Indonesia (1.11 MMT), Egypt (0.88 MMT) and Bangladesh (0.32 MMT).[3] The global tilapia industry and its associated value chains are currently estimated to be worth about US$ 7.9 billion.[8,9] Access to genetically improved elite strains of Nile tilapia (*Oreochromis niloticus*) is further fuelling the growth of the tilapia industry across the globe. Members of the genus *Oreochromis* are important not only for providing food and employment for local people, including women and youth, but also for earnings from domestic market and international export.[10–12] Today, Nile tilapia has become the third-most produced fish of all finfish species, representing a major source of affordable protein nutrients for multitude of consumers in many low- and middle-income countries (LMICs) across Asia, Africa, America and the Pacific.

Infectious diseases remain a serious bottleneck for aquaculture development, particularly in Asia where over 89% of the global production takes place.[3] Globally, disease-related losses in the aquaculture sector were estimated to exceed USD 6 billion in 2017.[13,14] Finfish aquaculture alone suffered annual losses ranging from USD 1.05 to USD 9.58 billion per year.[15,16] For many years, tilapia was perceived as hardy and disease resistant but this has changed in the face of intensification, climate change and global trade of live aquatic species, where global tilapia farming is now affected by serious disease problems caused by parasites (e.g., protozoan, monogenean), bacteria (e.g., *Streptococcus* spp., *Aeromonas* spp., *Edwardsiella* spp., *Flavobacterium columnare*, *Francisella orientalis*) and viruses (e.g., tilapia lake virus [TiLV], infectious spleen and kidney necrosis virus [ISKNV], tilapia parvovirus [TiPV] and nervous necrosis virus [NNV]) that are impacting the performance of the industry globally. The true economic cost of diseases in the tilapia industry is hard to estimate, but based on selected case studies[15,17–19] disease-related losses could run up to several billion dollars annually. For example, the value of 300,000 tonnes of tilapia lost due to disease caused by

Streptococcus spp. infections was estimated at USD 500 million.[20] Disease is also seen as a primary driver for increased use (misuse) of antimicrobials contributing to antimicrobial resistance (AMR) problems in aquatic food systems.[21–25]

Global outbreaks of Streptococcosis and recent outbreaks caused by TiLV and ISKNV in farmed and wild tilapia have drawn the attention of aquatic health specialists and policy makers worldwide to call for more research and better understanding of diseases and their management in tilapia aquaculture. Adoption of disease management practices such as routine diagnostics and biosecurity measures with other disease prevention approaches are going to be central to ensure sustainability of tilapia farming. Compared with high value salmonids and shrimp, the global research and development investment toward disease diagnostics and health management in low value but affordable species like tilapia, carps and catfish is less. As a result, adoption of effective health management and biosecurity practices relatively weak in LMIC undertaking farming of low value species.

Diagnostics may be defined as the determination of the cause or nature of a disease through the examination of signs, symptoms and diagnostic tests.[26] Diagnostic tests include both straightforward, pond-side methods and more advanced laboratory-based techniques requiring a high level of expertise and infrastructure. Disease diagnostics play three essential roles in aquaculture health management and disease control.[27,28] Firstly, diagnostics for screening healthy animals to ensure that they are not inapparent carriers of pathogens is aimed at disease prevention and is typically used to identify populations that have tested negative for specific pathogens as required for domestic or international translocation. This helps to limit the risk of disease transmission from farm to farm at national and international levels. Diagnostics play a crucial role in avoiding the transboundary transmission of a significant number of pathogens between countries and continents. Secondly, diagnostics have been used for routine health monitoring of farmed animals in order to detect infection/illness at an early stage. This facilitates timely intervention on the host–pathogen–environment complex to avoid a scenario of disease outbreak and substantial economic losses. Thirdly, diagnostics are used to diagnose diseases in animals that have clinical signs of illness. In this scenario, determining the cause(s) quickly and accurately is crucial for implementing appropriate management actions (e.g., treatment decisions, emergency harvest, etc.) to limit the negative impact on aquaculture farms in the short- and long-term. Diagnostics is particularly important in national disease monitoring programs, which provide the scientific foundation for development of national policies, emergency responses, risk management and biosecurity measures.[28–30] Such policies protects the sector from disease risks underpin international trade agreements in biological commodities.

The Snieszko circle,[26,31] also known as the epidemiological triad,[32,33] shows the relationship between the host, the pathogen and the environment in disease development (Figure 1a). However, in the triad, anthropogenic factors are incorporated into the environment circle of the Venn diagram which underplays their importance in the onset and outcomes of infectious disease, particularly in modern aquaculture. In 2013, Shields updated the triad to an epidemiological tetrad to reflect the significant anthropogenic drivers behind outbreaks of lobster diseases in Long Island Sound, The United States.[34] These included extensive eutrophication leading to hypoxia, exposure to metals and pesticides and various fisheries induced stressors.[34] Here we adapt the tetrad to reflect farmed rather than wild animal disease investigation, although there is substantial overlap (Figure 1b). There are many human impacts on farm animal health. These include actions of the farmer such as water management, animal handling, stocking practice, feed storage and feeding regimes.[35,36] There are directly connected actors such as feed companies, where diet provided to farms may not be optimally

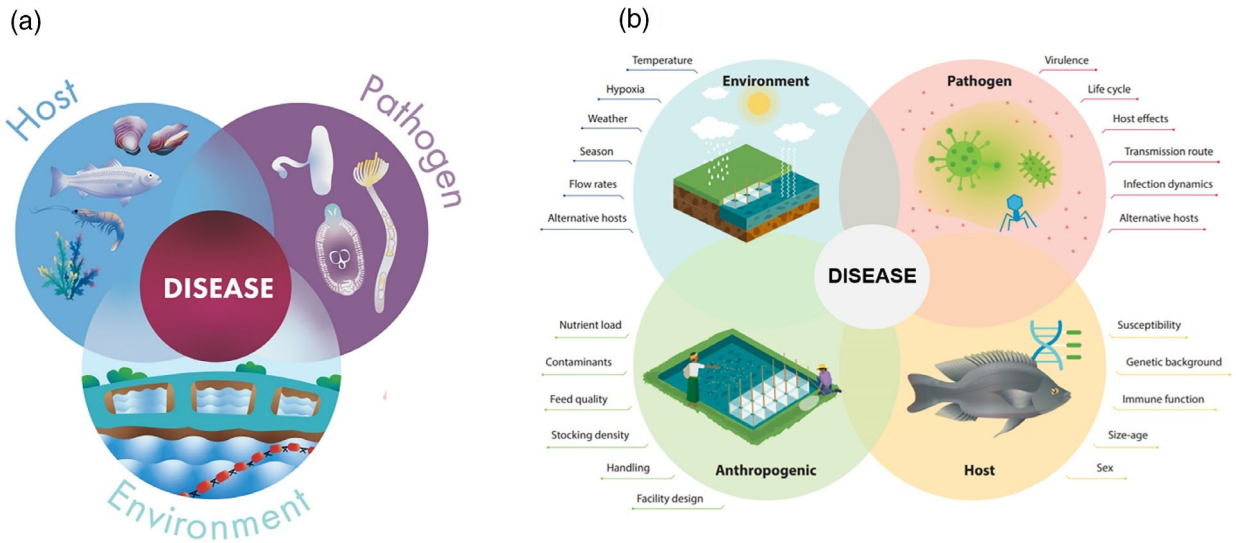

FIGURE 1 (a) The epidemiological triad[31] and (b) the epidemiological tetrad modified from Shields (2013).[34] The tetrad is based upon the original triad of Sniezsko,[31] but is modified to separate anthropogenic drivers of disease outbreaks from those that are purely environmental. This is an important consideration for disease investigation in fish farms where multiple stakeholders may have direct and indirect influence on farming conditions and consequently animal health. It highlights the importance of a broad based framework for diagnostic investigation and subsequent mitigation of disease. (Image A by M.G. Bondad-Reantaso and Paulo Padre, image B by A. C. Barnes and J. Delamare-Debouteville.)

formulated leading to immune compromise.[36] Finally, agriculture and urban water that are indirectly connected through shared water resource that may adversely impact the water available to the farm in terms of quantity (leading to inadequate water exchange and resulting hypoxia) and quality. Indeed, many pesticides and other pollutants are known to suppress the immune systems of aquatic organisms leading to disease.[34,37] The importance of the tetrad to disease diagnosis lies in the emphasis of a broad based investigation to establish cause and effect. The outcome of diagnosis, ultimately, is establishment of cause for effective treatment and prevention.

Diagnostics is an important element of a national strategy on aquatic animal health[38,39] (now called national aquatic organism health strategy) (Figure 2) and supports the other elements such as for example, policy, legislation and enforcement, risk analysis, pathogen list, border inspection, health certification, quarantine, farm-level biosecurity and health management, use of veterinary drugs, disease surveillance, emergency preparedness and contingency planning and others.

Availability of accurate diagnostic tools is an important criterion for listing of diseases in the OIE (currently known as the World Organisation for Animal Health, WOAH) Aquatic Animal Health Code.[40] Article 1.2.1 of the WOAH Aquatic Animal Health Code lists four criteria for listing an aquatic animal disease. These are: (i) significant production losses, negative affect on wild populations, zoonotic; (ii) infectious aetiology proven, strong association; (iii) capacity for international spread and (iv) diagnostic methods exist.

Diagnostic testing is an essential part (checklist no. 6) of a 12-point surveillance checklist for surveillance of diseases of aquatic organisms.[28] The choice of diagnostic technique needs to account for the following:

- analytical sensitivity which refers to the limit of detection for a disease agent
- analytical specificity which refers to the ability to distinguish the targeted disease agent from another
- diagnostic sensitivity which refers to the probability of test to correctly identify diseased individuals
- diagnostic specificity which refers to the probability of test to correctly identify non-diseased individuals

Quality assurance of a diagnostic system is also an essential part (checklist no. 11) of the surveillance checklist. Diagnostic laboratories that support surveillance could be any accredited laboratory recognized by the competent authority as having the appropriate technical competence in disease diagnostic work. Thus, proficiency ring tests, accreditation and analytical methods are all essential components of an overall quality assurance system.[28] ISO 17025 is the accepted international standard by which laboratories are accredited as being technically competent for specific diagnostic analyses.

Due to their significant benefits in terms of short turnaround time, high specificity and sensitivity, molecular diagnostic methods (e.g., polymerase chain reaction [PCR], quantitative real-time PCR [qPCR], digital droplet PCR [dPCR], loop-mediated isothermal amplification [LAMP], recombinase polymerase amplification [RPA] and others) have emerged as important technologies for improving disease diagnosis in aquaculture. Disease diagnosis in aquaculture was mainly reliant on clinical observation, rapid microscopic inspection by wet-mount and/or quick staining of smears or imprinted tissue, histopathology and culturing of infectious agents prior to the expansion and adoption of molecular

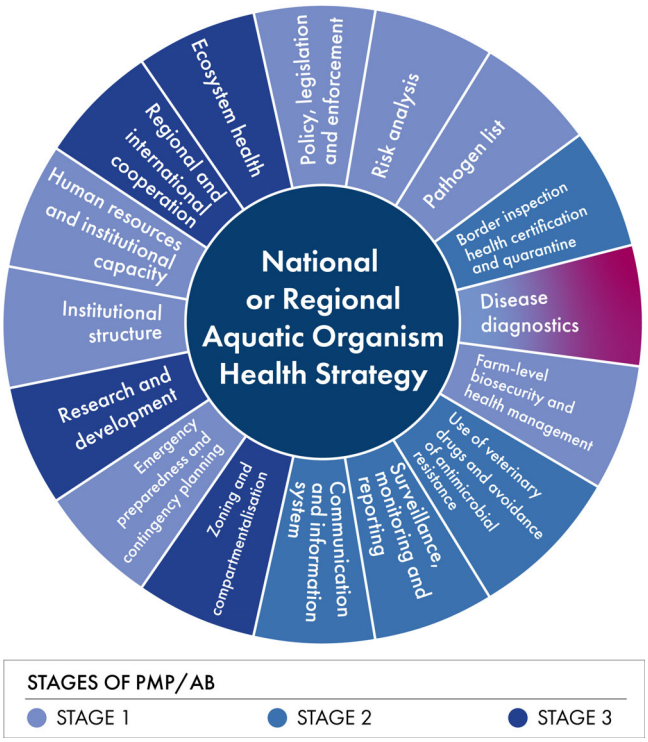

FIGURE 2 Important elements or components of a national aquatic organism health strategy where each element is not a stand-alone component but rather supports each other. (Image by M.G. Bondad-Reantaso and Paulo Padre)

methods in the early 2000s.[41,42] Importantly, many infectious diseases in fish and shrimp are initially discovered and diagnosed based on Level I (see Section 2, below) gross clinical observations and traditional histopathology (Level II). For example, disease caused by TiLV was detected and defined for the first time as syncytial hepatitis of tilapia (SHT) based on a pathognomonic lesion identified in the liver of sick tilapia using classical histology.[43] Similarly, an unknown viral disease, scale drop syndrome, in Asian sea bass was discovered based on gross pathology and histopathological findings of viral inclusion.[44] The shrimp microsporidian *Enterocytozoon hepatopenaei* (EHP) was discovered as a novel microsporidian based on histopathological observation of cytoplasmic spores and multinucleated plasmodia in the shrimp hepatopancreas.[45] In these cases, the disease was identified histopathologically before its causative agent became known to science and before any molecular diagnostic procedures were available for the causative agents. Recent widespread use of molecular diagnostics (Level III) in aquaculture has shifted the focus of diagnostic application away from observational approaches (Level I and II). However, there has been some evidence indicating that molecular diagnostic methods, including those from published papers, The WOAH recommended protocols and commercial kits, sometimes give false-positive results (see Refs. [46–48]). Clinical observations and microscopic examination, on the other hand, are useful for presumptive diagnostics, which guides the choice of the appropriate level II diagnostic test(s) and serves as a clinical judgment in diagnostic error(s). Thus, disease diagnostics should involve a combination of fundamental and sophisticated procedures, including macroscopic, microscopic and molecular investigation, to achieve accurate and meaningful results. In this review, we therefore revisit and emphasize the necessity for fundamental diagnostic procedures for tilapia diseases. Furthermore, current and emerging molecular diagnostic methods are discussed, and their future prospects are critically addressed.

2 | BACK TO BASICS: THREE LEVELS OF DIAGNOSTICS FOR INFECTIOUS DISEASES IN AQUACULTURE

Disease diagnostics is the procedure by which the causative agent of an infectious disease is identified. The Food and Agriculture Organization of the United Nations (FAO) and the Network of Aquaculture Centers in Asia and the Pacific (NACA) have long promoted the use of levels I, II and III for disease diagnosis[39,49] (Figure 3). The principle being that none of the three diagnostic levels function in isolation. They form a continuum of observations (Figure 3) with strong linkages needed for accurate and rapid diagnosis (e.g., for general health surveillance, health certification of import stock and to reduce the risk of disease introduction into disease-free areas) so that appropriate and effective management measures can be rapidly applied.

Level I provides the foundation and is the basis for accurate interpretation of results obtained from Levels II and III laboratory findings. It also sets the foundation for 'presumptive' and 'confirmatory' diagnostic test reporting. Presumptive tests establish if a sample is not infected by a pathogen, or that it is likely infected by a pathogen. In the latter case, it may remain presumptive where the test cannot distinguish pathogenicity (just presence/absence) or the exact identity of the pathogen (e.g., endemic from exotic strain/species). Confirmatory tests are then required to confirm (or refute) the presumptive analysis. Level I may be sufficient for recurrent, pathognomonic

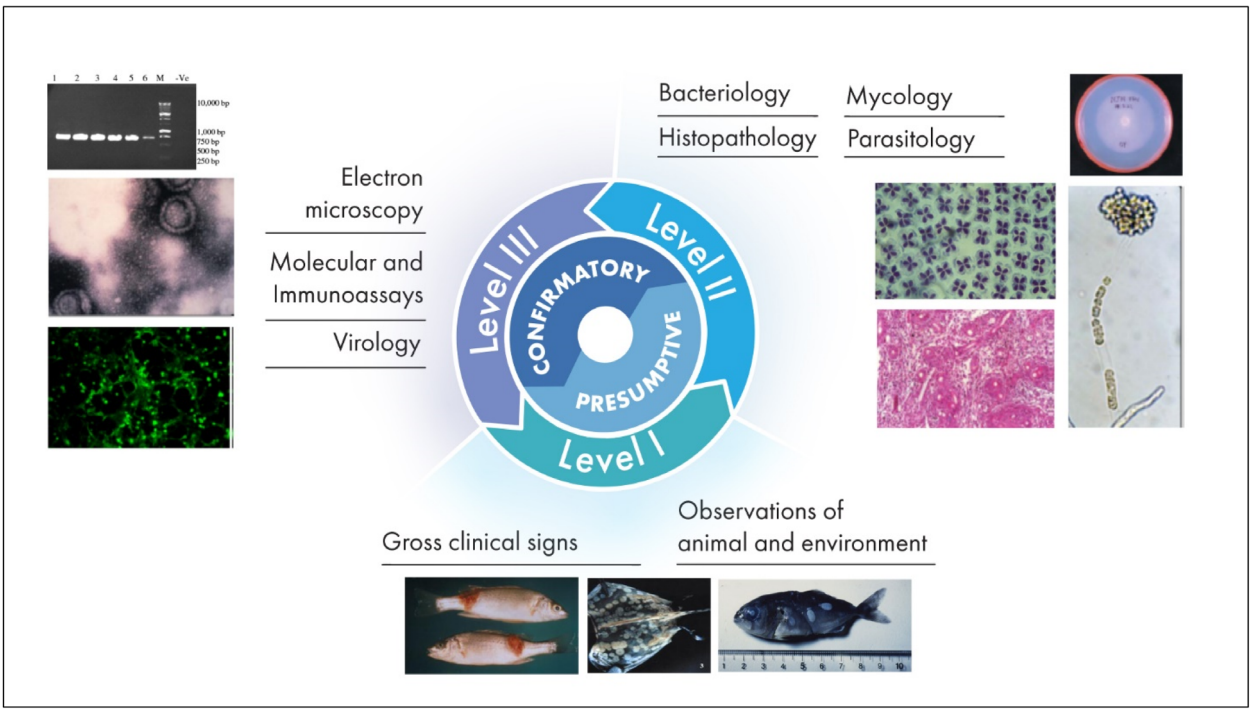

FIGURE 3 The three diagnostic levels (I, II and III) are a continuum of observations; each level builds on the other and contributes valuable data and information to build a diagnostic case for optimum diagnostic accuracy. (Image by M.G. Bondad-Reantaso.)

(i.e., clinical signs are specific to a particular pathogen or environmental stressor) infection. However, confirmatory diagnoses, most commonly, require Level II or III equipment and expertise to distinguish significant pathogens from more benign, infectious species or strains.

Level II laboratories include the equipment and experienced personnel to undertake analyses that can detect and/or identify a range of pathogens. Level II laboratory personnel can perform parasitology, histopathology, bacteriology and mycology examinations. Level II, particularly histopathology, remains the gold standard, especially for unknown and emerging diseases.

Level III diagnostics encompass techniques that target a specialized pathogen or group of pathogens or require highly specialized equipment. Level III laboratories are highly specialized and many such laboratories are accredited nationally or by the WOAH as 'Reference Laboratories'.[50] These laboratories can also be used to confirm disease-freedom to reinforce national health certification for import-export purposes. Use of Level III techniques support Koch's postulate to prove that a particular organism causes a particular infectious disease is important, especially for first time diagnosis of an unknown disease in a country.

Level III diagnostics rarely consider interactions at the host–pathogen interface (pathogenicity) as it relies on detection of molecular signals of these interactions and does not take environmental parameters into account. Thus, any correlation falls on linkage with Level I or II diagnostic observations. The increasing availability of field rapid test kits has been a major advantage for field extension officers, aquatic animal health specialists and farm veterinarians, but brings into play the risk of false/negative results without adequate user training and interpretation. Thus, the importance of conclusive diagnoses being based on more than a single test cannot be under-estimated, and is now clearly outlined by the WOAH[50] for their listed diseases. Accuracy of results is significantly augmented by two or more consistent results, especially of new or previously unknown disease outbreaks.

Three levels of diagnostics can be flexibly applied for infectious diseases of tilapia including bacterial, viral, parasitic and fungal diseases (Figures 4 and 5). At level I, presumptive diagnostics comprises observation of abnormal behaviours, clinical presentation, historical record, environmental parameters and preservation of samples for subsequent analyses in levels II and III. Fish from an affected pond/cage usually exhibit abnormal swimming behaviour, such as failure to school, with separation of sick individuals in the corner or bottom of the pond or cage. Diseased fish may show pale colouration, dark colouration, reddish gill opercula, skin haemorrhage and scale protrusion. Internally, clinically sick fish can exhibit a pale, watery and necrotic liver, accumulation of yellow ascetic liquid in the peritoneal cavity and gas in the intestine.[11,43,51] At level II, presence of syncytial hepatitis is considered pathognomonic for TiLV infection, while intracytoplasmic inclusion bodies may also, occasionally, be observed.[43] More recently, liver tissue smears stained with Haematoxylin and Eosin (H and E) has been found to be a simple and effective approach for rapid screening of syncytial hepatitis (or giant cells) (experience from HT Dong, see Figure 4). Several molecular techniques including reverse transcription PCR (RT-PCR), nested or semi-nested RT-PCR, RT-qPCR, RT-LAMP and in situ hybridization (ISH)[11,47,52–57] culturing of virus using cell line,[11,56,58] TEM[11,43,59] and enzyme-linked immunosorbent assay (ELISA)[60] have been applied for diagnostics of TiLV at level III.

Similarly, bacterial diseases (e.g., Streptococcosis, Columnaris, Edwardsiellosis, Francisellosis and Aeromonasis) in tilapia can be

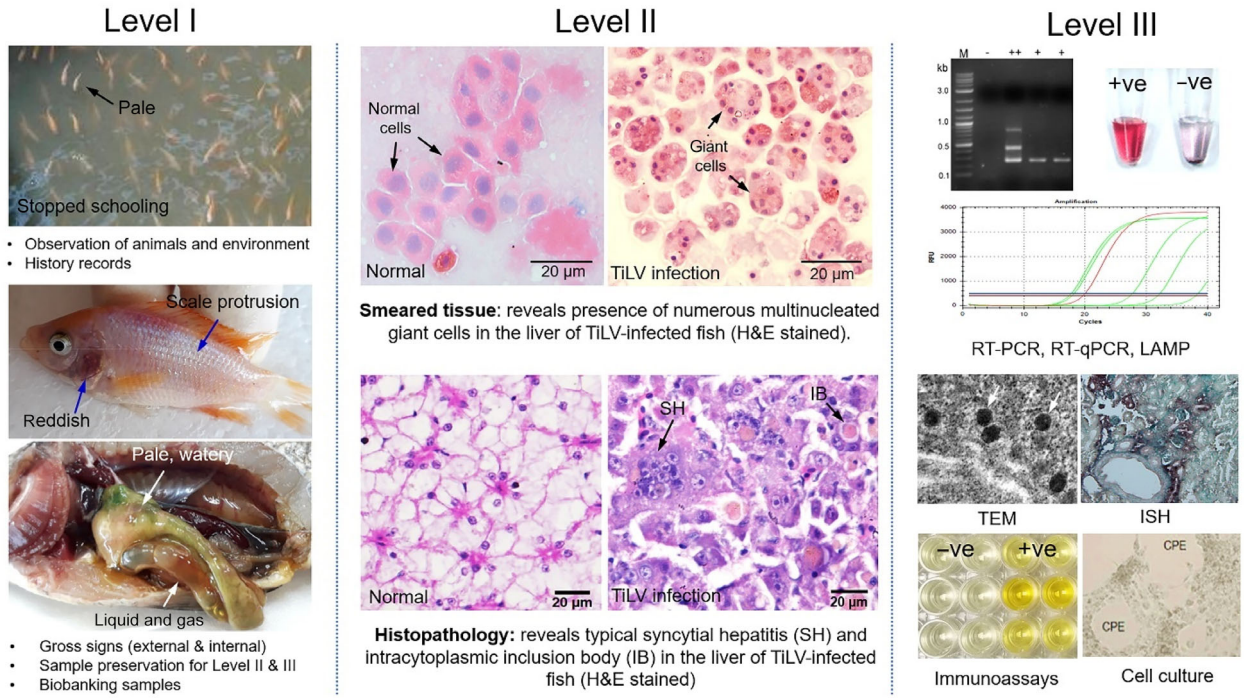

FIGURE 4 Illustration of three levels of disease diagnostics for tilapia lake virus disease in tilapia. (Image by H.T. Dong.)

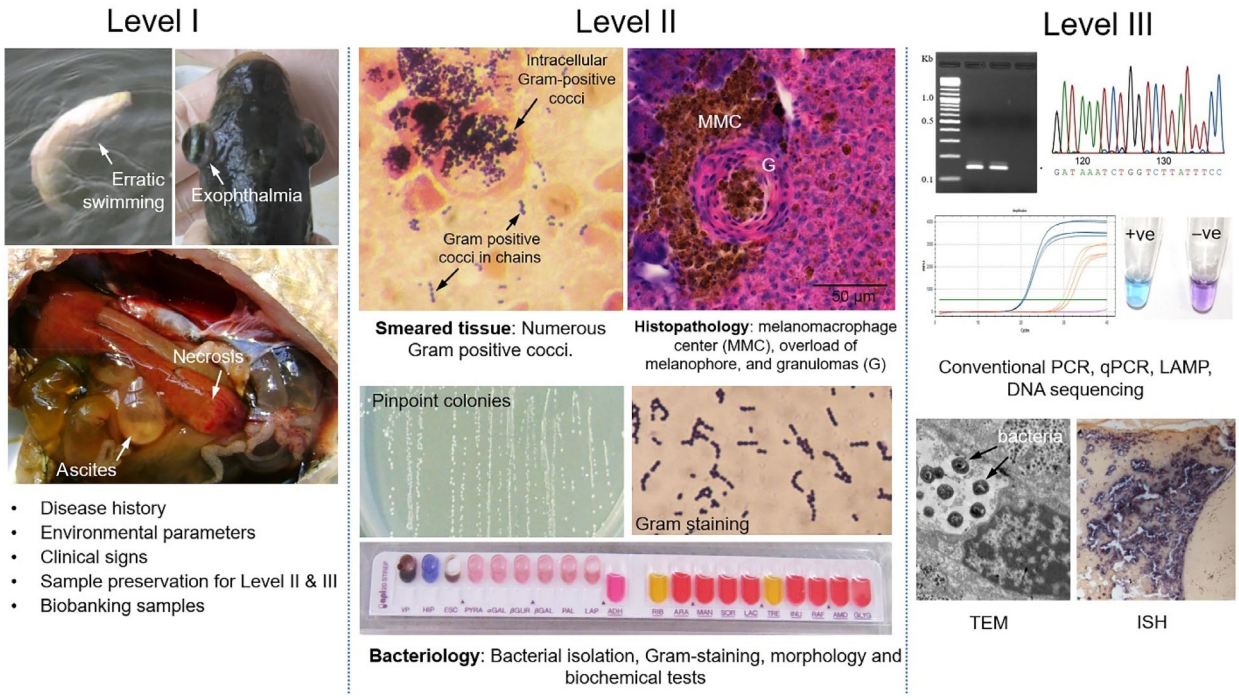

FIGURE 5 Example of three levels of disease diagnostics for Streptococcosis in tilapia. (Image by H.T. Dong.)

presumptively diagnosed using levels I and II, including clinical signs, wet-mount and smeared tissues stained with Giemsa or Gram stain. Further analysis using level II (histopathology, bacterial culture, biochemical screens) and level III (molecular methods) approaches is usually employed for confirmatory diagnosis of suspected bacterial diseases.[61,62] An example of three diagnostic levels in the context of Streptococcosis is shown in Figure 5. At level I, erratic swimming and exophthalmia were considered important clinical signs for presumptive diagnostic of *Streptococcus* sp. infection. Internally, diseased fish presented with ascites, accumulation of liquid in the intestine and dark brown and necrotic liver. At Level II, Gram or Giemsa-stained tissue smears from head kidney was useful for visualization of extra- and intracellular Gram-positive cocci. Histopathologically, diseased fish exhibited increasing melanomacrophage centres and granulomatous inflammation with overload of melanophores in the liver. *Streptococcus* sp. could be isolated from diseased fish using general culture medium such as blood agar (BA), nutrient agar (NA), tryptic soy agar (TSA) or brain heart infusion agar (BHIA), morphologically identified by microscopy of Gram stained samples and biochemically characterized by commercial processes such as API 20 Strep or Vitek. Several methods can be employed at level III for confirmatory diagnostic of Streptococcosis including conventional specific PCR,[63] qPCR,[64] LAMP,[65–67] sequencing of 16S rRNA,[68] ISH and TEM.

In reality, it is unlikely that disease outbreaks in tilapia farms in LMIC are currently diagnosed in a timely manner by rigorous diagnostic tests. Therefore, level I diagnostics should be considered through observation of clinical signs,[69] case history records, outbreak description as part of the syndromic surveillance to support early presumptive diagnosis and also to make informed evidence-based decisions on appropriate further sampling and diagnostic approaches, as well as immediate management actions. Preservation of biological samples (biobanking) might be useful for retrospective diagnostics as well as epidemiology and evolution of infectious agents.[70–72] In the context of tilapia disease diagnosis, the term 'biobanking' refers to the systematic preservation of biological materials in a suitable manner for later examination using advanced diagnostic methods. Fixed tissues or blood (e.g., in ethanol 95% or RNA later for molecular testing) and nonfixed frozen tissues or serum (e.g., storing at minus 80°C or liquid nitrogen for later recovery of infectious agents) are examples of these samples. The biological samples also include pathogens (isolates/strains) recovered from diseased animals, extracted genetic materials (DNA or RNA) and paraffin-embedded samples. Appropriate biobanked samples provide the necessary materials for interconnecting three diagnostic levels (I, II and III) which are required to progress from presumptive to conclusive diagnoses.

3 | CURRENT AND EMERGING MOLECULAR DIAGNOSTIC TECHNOLOGIES

The field of molecular diagnostics has, in recent years, developed rapidly and contributed substantially to our ability to detect and identify microbial pathogens of aquatic organisms, most importantly the detection of sub-clinical carriers. Various nucleic acid-based amplification techniques are commonly used in detecting aquatic pathogens, including conventional PCR, qPCR, dPCR, LAMP and CRISPR. The strengths and limits of each technology, and their current and potential application for disease diagnosis in tilapia aquaculture is discussed below.

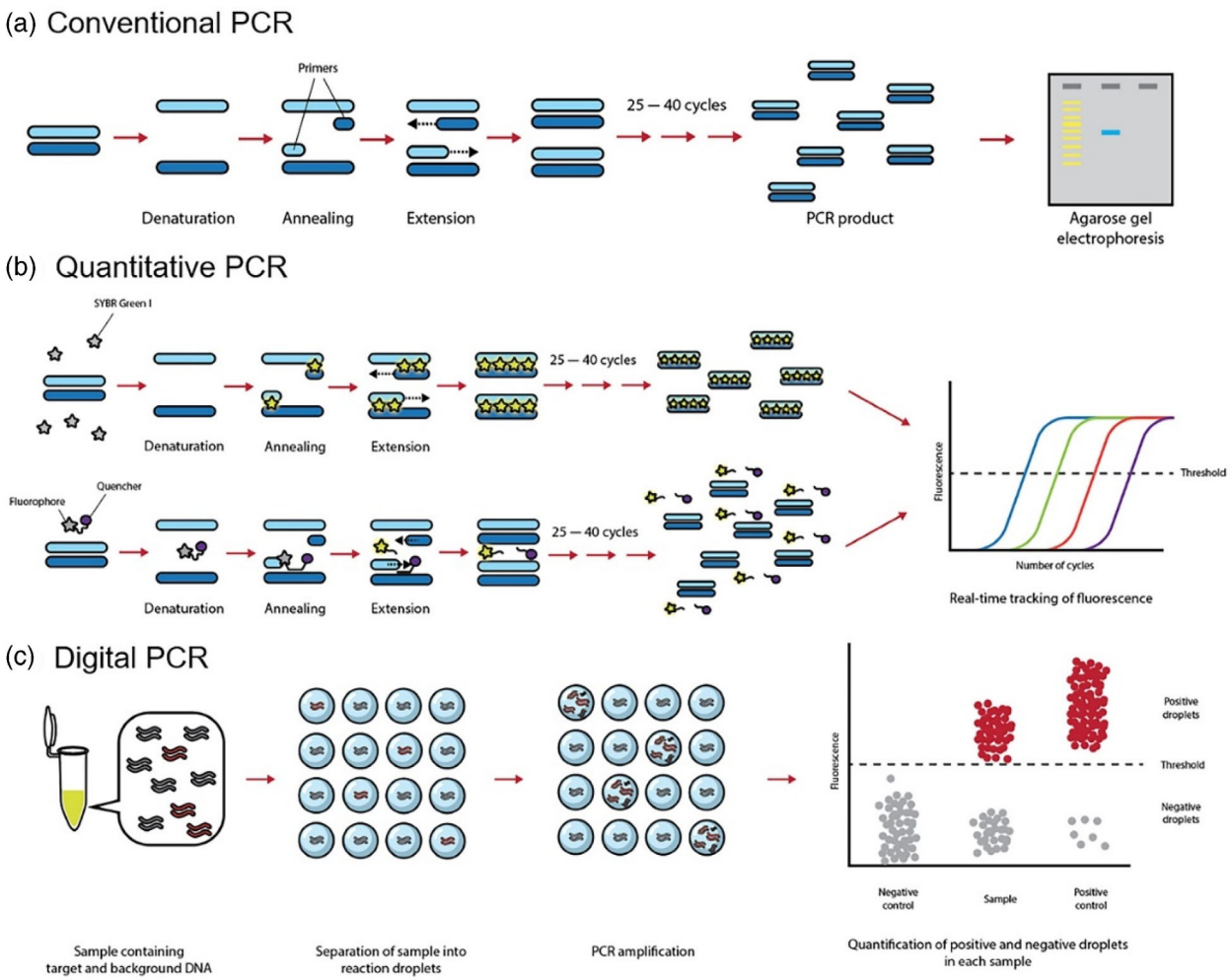

FIGURE 6 Illustrations depicting the backbones of conventional PCR (a), quantitative PCR (b) and digital PCR (c). (Images by T. Chaijarasphong and H.T. Dong.)

3.1 | Polymerase chain reaction

3.1.1 | Conventional polymerase chain reaction

Polymerase chain reaction (PCR) is a method that employs a thermostable polymerase to amplify a specific region of DNA defined by a pair of primers. PCR relies on thermal cycling; the DNA templates are exposed to repeated cycles of heating and cooling to permit DNA melting, annealing of the primers and DNA synthesis by the polymerase. This reaction generates large numbers of DNA synthetic copies from a small amount of DNA template (Figure 6a). When the reaction is 100% efficient, approximately 10^9 copies of DNA target can be produced per template after 30 cycles. As primer and deoxynucleotide triphosphate (dNTP) are consumed during the reaction, single-step PCR has limited sensitivity. Nested PCR, two successive PCR reactions, using second round primers specific to the first-round amplicon, provides increased sensitivity and specificity, and has been developed for detecting pathogens in sub-clinically infected animals. The PCR procedure involves extraction of DNA (or RNA) from host tissue samples, followed by amplification of target DNA. A Taq polymerase, a major component of PCR, will not work on an RNA template, so PCR cannot be used to directly amplify an RNA molecule. For detecting RNA viruses, extracted RNA must be first transcribed into its complementary DNA (cDNA) by the enzyme reverse transcriptase (RT). This method of RNA amplification is called reverse transcriptase-polymerase chain reaction (RT-PCR).

Advantages of PCR-based diagnosis include their high sensitivity and specificity, rapid turnaround, elimination of the need for prior isolation or culturing of microorganisms and relatively low cost. The method is especially useful for detecting pathogens in inapparent infected individuals and in identifying pathogens that are unculturable, such as shrimp or molluscan viruses, or difficult to culture, such as intracellular bacteria. However, PCR requires trained technicians for optimization and reliable diagnostic results, along with well-equipped facilities with strict protocols for nucleic acid extraction and processing. PCR is susceptible to contamination and requires strict consistency of procedures for high throughput automation.

Conventional end-point PCR/RT-PCR (including single, semi-nested, nested PCR, duplex and multiplex PCR) has been commonly used for the detection of infectious pathogens in tilapia such as TiLV,[11,47,57,73] TiPV,[74] NNV,[75] *S. agalactiae*,[76,77] *S. iniae*.[78,79] In tilapia, *S. agalactiae* and *S. iniae* are the two most frequently detected

bacteria that cause streptococcosis. Both cause similar clinical signs, thus a duplex PCR using two primer pairs and a differential PCR using a single primer pair were developed for detecting and differentiating these two bacteria.[63,80] Multiplex PCR was also developed for serotyping of *S. agalactiae*.[81]

PCR methods have also been developed for detecting *F. orientalis* and *F. columnare*,[82,83] which are fastidious bacteria that require time-consuming, complex culture media and biochemical assays for non-molecular infection diagnosis in tilapia.

Edwardsiellosis and motile *Aeromonas* septicemia (MAS) are among the most prevalent bacteria detected following mortality in freshwater fish, including tilapia. PCR-based methods were developed for detecting of *E. ictaluri* and *E. tarda*[84] and applied for tilapia.[85] For *Aeromonas* bacteria, PCRs targeting the virulence-associated genes, hemolysin and aerolysin, were developed to identify *A. hydrophila* isolated from tilapia with MAS.[86]

3.1.2 | Quantitative real-time PCR

Quantitative real-time PCR (qPCR) is a well-established method for diagnosis of aquatic animal diseases. Amplification of target nucleic acids can be detected in real-time during PCR through the use of either sequence-specific fluorescent-labelled oligonucleotide probes (e.g., TaqMan), or sequence-independent fluorescent dyes (e.g., SYBR Green I).[87] The presence and quantity of a target DNA can be determined by its cycle threshold (Ct) value, which corresponds to the cycle number when the fluorescence level is significantly above a pre-defined, experimentally determined threshold (Figure 6b). This method eliminates post-PCR gel electrophoresis and thus reduces the risk of cross-contamination between samples during loading of the gel. Usually, a cut-off Ct value is determined based on a limit of detection established experimentally. This helps to eliminate false positives based on non-specific amplifications, and the test is interpreted as positive if the Ct value is less than the cut-off Ct. If the Ct value is greater than the cut-off value (i.e., below the limit of detection), the test may be interpreted as negative.

Quantitative real-time PCR measures fluorescence intensity and can be used to quantify the number of copies of target nucleic acids present in a tissue sample to determine the viral (or other microorganism) loads. Quantification of a specific virus in tissues of infected animals is one of the most important means of monitoring the progression of a disease. Cell culture-based methods of quantifying pathogens are time-consuming and not applicable to some aquatic organisms, such as shrimp, for which cell culture systems have not been developed. qPCR has the advantages of rapid, high-throughput and a wide dynamic range (7–8 $\log_{10}$) for quantification; it can be multiplexed to detect several targets in a single reaction.

RT-qPCR or qPCR procedures have been developed and optimized for the detection and quantification of viral or bacterial loads in infected tilapia. Target pathogens include TiLV,[55,56,88] ISKNV,[89] TiPV,[74] *S. agalactiae*[64,90] and *F. orientalis*.[91–93] Several multiplex TaqMan qPCR assays have also been developed to detect and quantify three to four pathogen species in a single PCR test, such as *F. orientalis*, *S. iniae* and *S. agalactiae*[94]; *A. hydrophila*, *A. veronii* and *A. schubertii*[95]; and *E. ictaluri*, *E. tarda*, *E. anguillarum* and *E. piscicida*.[96]

3.1.3 | Digital PCR

Digital PCR (dPCR) uses the same analytical process as qPCR, but is used to quantify the absolute number of target DNA molecules.[97] In dPCR, the DNA template and reagents (identical to the qPCR reaction mixture, including pathogen-specific primers and probe) are mixed and then partitioned, either in emulsion droplets or in wells, on a nanofluidics chip. dPCR amplification is then performed on each of the partitions. At the end of the dPCR, each partition is read, and the absolute quantification of DNA template is calculated with Poisson statistical analysis. The process is 'digital' in that each partition is scored as either 1 (positive) or 0 (negative) (Figure 6c). It is important that the DNA template be adequately diluted, as most partitions contain one or no target DNA molecules.[98,99]

Digital PCR has advantages over qPCR in that dPCR does not require a standard curve for quantifying the DNA template and provides more accurate quantitative results, because the presence of PCR inhibitors has little effect. There are two disadvantages to dPCR: (1) it is laborious and has a lower throughput, and (2) it has a smaller dynamic range than qPCR, so samples need to be diluted within a specific range to generate accurate results.

Digital PCR is relatively new to aquaculture so has only been applied to few fish pathogens. dPCR methods are available for ISKNV[100] and *S. agalactiae*.[101] The detection limit of ISKNV dPCR was determined to be 1.5 copies/μl, which is substantially lower than the 34 copies/μl of a TaqMan qPCR. This assay was used to detect ISKNV in mandarin fish (*Siniperca chuatsi*) and shown to have a higher positive rate (65%) than that of qPCR (30%).[100] Similarly, the latter method was developed for absolute enumeration of *S. agalactiae* in tilapia tissue which is more sensitive than conventional plate count method and qPCR.[101] These results suggest that dPCR presents a promising diagnostic platform for other tilapia pathogens.

3.2 | Isothermal amplification

Isothermal amplification methods (IAM) present a powerful class of nucleic acid detection analytics that provide streamlined workflows and rapid turnaround times, while preserving the diagnostic merits of conventional PCR. By using polymerases capable of replicating nucleic acids at a constant temperature, IAM avoid the thermal cycling associated with PCR, making them ideal for on-site diagnosis in areas lacking scientific resources and manpower. To date, a plethora of IAM have been developed and implemented with varying degrees of success, including loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), nucleic acid sequence-based amplification (NASBA), helicase-dependent amplification (HDA), rolling circle amplification (RCA) and cross-priming amplification (CPA). This section of the review will focus on two IAM that show potential for rapid detection of tilapia diseases: (1) LAMP, by far the most frequently used

IAM, and (2) RPA, which has grown in use over the last decade as a result of its improvements over LAMP's shortcomings. Additionally, we will discuss the combination of isothermal amplification and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) diagnostic analyses, which open up new potential applications currently not feasible with IAM alone.

3.2.1 | Loop-mediated isothermal amplification

A typical loop-mediated isothermal amplification (LAMP) reaction consists of a DNA template, four to six primers targeting six to eight distinct regions along the target DNA, and the large fragment of *Bacillus stearothermophilus* (*Bst*) strand-displacing DNA polymerase.[102,103] The reaction is typically completed within an hour at a temperature of 60°C–65°C and progresses exponentially through characteristic, dumbbell-shaped DNA intermediates, eventually generating concatemers of various sizes harbouring the target sequence (Figure 7a). This size heterogeneity of LAMP products manifests as ladder-like bands when analysed by agarose gel electrophoresis. To detect RNA viruses such as TiLV, reverse transcription step by reverse transcriptase (RT) must be incorporated into the procedure to produce cDNA from the RNA target prior to LAMP. Alternatively, a newer generation of *Bst* polymerase, *Bst* 3.0, that shows high reverse transcriptase activity, could be utilized for single-enzyme RT-LAMP protocols.[104] LAMP platforms have been described for the detection of tilapia pathogens, including *Streptococcus agalactiae*,[65–67] *Flavobacterium columnare*,[105] *Shewanella putrifaciens*,[106] ISKNV[107] and TiLV.[54,108,109] These applications exhibit high sensitivity and specificity for their respective targets, with the lowest limit of detection reported at 1 viral copy per reaction, approximately 100 times lower than that typically obtained using conventional PCR.[54]

While different LAMP assays use similar nucleic acid amplification procedures, they vary in their visualization methods, which range from being laboratory oriented to field compatible. Agarose gel electrophoresis, for example, is commonly employed in laboratory settings, but it is time-consuming and requires extensive reagent preparation and handling. While it can be used to validate amplicon size in PCR, this advantage is lost in LAMP due to the products' ladder-like appearance precluding direct size comparison. To further streamline detection, colorimetric dyes such as SYBR Green,[109] calcein[105,106,108] and hydroxynapthol blue[107] can be incorporated to LAMP reactions. The use of these reporters enhances field deployability of the assay, but they are sequence-independent and

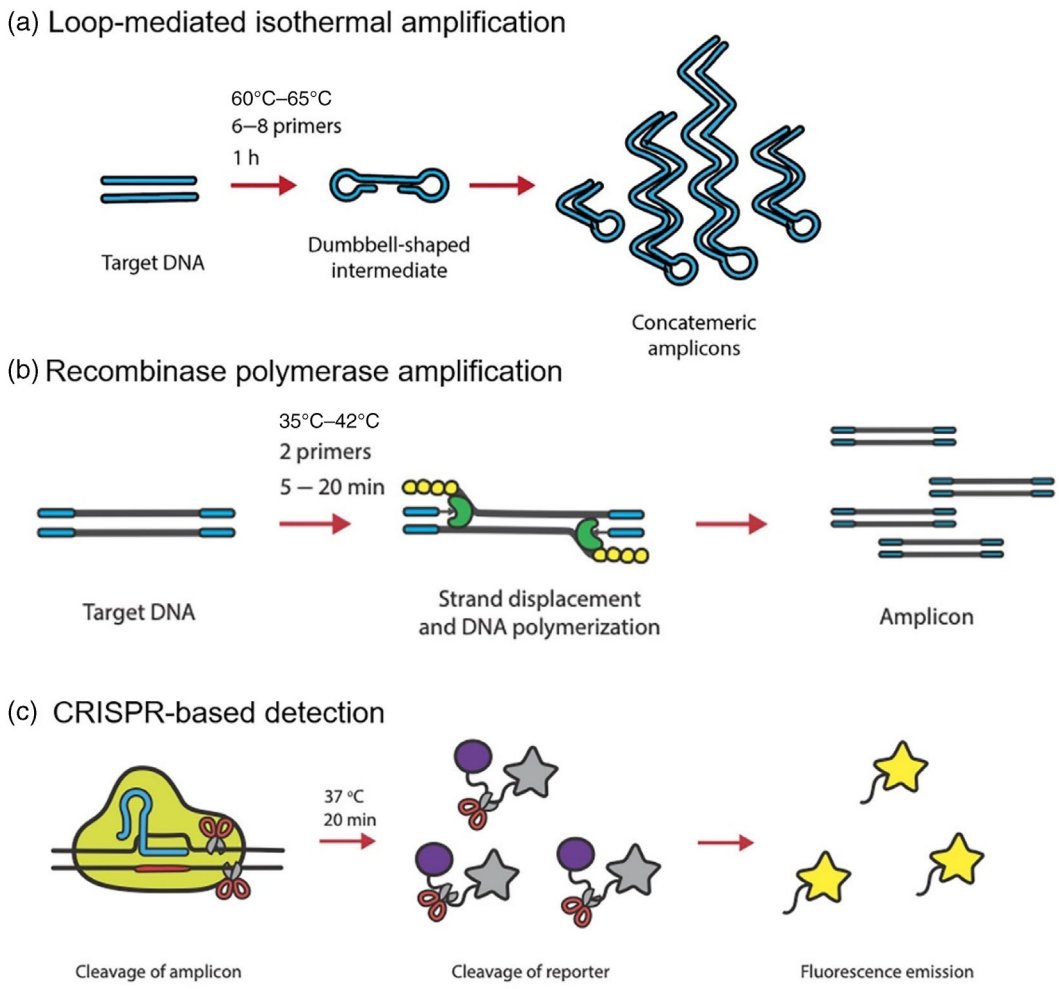

FIGURE 7 Illustrations depicting the backbones of RPA (a), LAMP (b) and CRISPR-based detection (c). (Images by T. Chaijarasphong.)

thus incapable of discriminating between specific and spurious amplification products. To exclude non-specific amplicons, sequence-specific hybridization probes, such as gold nanoparticles (AuNP) coated with single-stranded DNA (ssDNA), can be used.[54] Upon denaturing and reannealing of LAMP products, the gold-conjugated ssDNA hybridizes to its complementary region in the valid amplicon, preventing AuNP from aggregating in the presence of high salts. As a result, a positive reaction retains the pink colour of dispersed AuNP, while a negative sample precipitates AuNP and loses its solution colour.

3.2.2 | Recombinase polymerase amplification

While LAMP has substantially improved the convenience of tilapia disease detection, the technique still faces a number of limitations including the large number of primers required, which increases the likelihood of primer–dimer formation, and the reaction temperature that, while constant, is still sufficiently high to require a heating device.[110] In comparison, a relatively recent IAM called recombinase polymerase amplification (RPA) requires a relatively low temperature between 35° and 42°C that can be supplied instrument-free, has a short reaction time of 5–20 min, and requires only two primers, similar to PCR.[111] This assay relies on a bacterial recombinase protein to partially unwind the target DNA duplex and enable primer annealing to the complementary regions.[112] The reaction also contains single-stranded DNA-binding proteins that sequester the displaced DNA strand and prevent it from reannealing (Figure 7b). With primers in place, DNA polymerase initiates exponential DNA amplification and generates a large amount of daughter DNA that can be visualized by agarose gel electrophoresis, fluorescence or lateral flow detection.[113,114] To detect RNA, a preincubation step with reverse transcriptase at 42°C can be directly incorporated into an RPA reaction, yielding an RT-RPA workflow. Thus far, RPA methods have been used to detect a number of pathogens affecting tilapia, such as *Aeromonas hydrophila*, *Flavobacterium columnare* and *Francisella noatunensis* subsp. *orientalis*, with an analytical sensitivity of up to 15 DNA copies per reaction.[115–118]

3.2.3 | CRISPR detection

Due to their low reaction temperatures, IAM like LAMP and RPA are intrinsically susceptible to primer dimer formation and non-specific amplification. Additionally, the sensitivity of the assays is highly target-dependent, with challenging targets needing extensive, iterative optimization to enhance sensitivity. Integrated with CRISPR detection, specificity and sensitivity of IAM can be raised in a plug-and-play manner.[119,120] The CRISPR detection method begins with an RNA-guided CRISPR-associated protein (Cas) endonuclease, such as Cas12a or Cas13a, recognizing and cleaving the target nucleic acid (e.g., IAM amplicon). This on-target cleavage induces a conformational change in the Cas protein, causing it to indiscriminately digest the ssDNA (in case of Cas12a) or ssRNA (in case of Cas13a) that connects a fluorophore and its quencher in the synthetic reporter, resulting in unquenching and consequent fluorescence emission[121,122] (Figure 7c). Thus, the presence of the positive amplicon is converted into a fluorescent signal observable by the naked eye, or, with some modification, a colorimetric signal on a lateral flow dipstick.[123,124] It should be noted that Cas13a, which exclusively targets ssRNA, requires the addition of RNA polymerase and nucleoside triphosphates (NTP) as well as the presence of a promoter sequence in one of the IAM primers to allow transcription. This CRISPR detection step may be preceded with practically any IAM, although RPA is most commonly chosen due to its optimal temperature being close to that of Cas proteins (37°C).[125] On the other hand, the choice of Cas proteins is restricted to a small number of Cas homologues capable of carrying out reporter cleavage in the manner described above.[119,120,126] Indeed, Cas9, the most widely used homologue for genome editing, lacks nonspecific secondary cleavage activity so cannot readily be repurposed for diagnostic applications.[119,127,128]

Along with providing several modes of simple visual detection, integration with CRISPR may improve the sensitivity and specificity of IAM. The diagnostic Cas endonucleases are capable of increasing sensitivity owing to their multiple-turnover kinetics, whereby the cleavage of a single target DNA/RNA molecule activates Cas protein for digestion of several reporter molecules, resulting in signal amplification.[119,123] Nonetheless, this sensitivity enhancement effect is not always observed and is more frequently found with Cas13a than Cas12a, presumably due to the superior reporter cleavage kinetics of the former.[124,129–131] In terms of increasing specificity, by tailoring the CRISPR assay to target an area within the correct amplicon, it is possible to filter out nonspecific amplification products from IAM.[129] Moreover, Cas endonucleases are exceptionally stringent in their target recognition—a 2-bp mismatch between guide RNA and target nucleic acid has been shown to drastically reduce the cleavage activity.[120,132] This low mismatch tolerance can be used to genotype closely related pathogen strains whose sequences may be too similar for traditional PCR or IAM alone to differentiate. CRISPR detection, therefore, may allow for easy identification of geographical isolates or genotypes of RNA viruses such as TiLV, which may grow more diverse in sequence and virulence in the future due to their fast mutation rates. While CRISPR detection has been extensively applied to high-impact pathogens such as SARS-CoV-2, it has not yet been harnessed for disease detection in tilapia, highlighting an untapped opportunity for improving the efficacy and utility of the present diagnostic toolbox.[133,134]

3.3 | Lateral flow immunoassays

Although nucleic acid detection approaches are highly sensitive and specific, they are limited by long processing time, extensive liquid handling and the requirement for scientific instruments. While IAM have simplified overall procedures, some liquid handling and wait time remain necessary. In comparison, lateral flow immunoassays (LFIA) allow the user to simply apply the analyte to a ready-to-use strip and

wait for 5–10 min before reading the result, which is colorimetric and interpretable by eye. The analytic materials also have long shelf-life and can be stored at room temperature. Therefore, despite their generally lower sensitivity and specificity than comparable nucleic acid detection technologies,[135–138] the convenience of LFIA greatly aid screening of diseases and presumptive disease diagnosis in tilapia, as well as adoption by stakeholders who may be hesitant to use more laborious, time-consuming, diagnostic platforms.

To perform an LFIA, the sample must first be isolated from the source specimen. The extraction protocol varies depending on the target organ, but generally involves briefly homogenizing the tissue in a lysis buffer and collecting the supernatant.[139,140] The supernatant is then applied to a sample pad on a membrane-bound strip, before it is immersed in a running buffer. Alternatively, some LFIA kits use the lysis buffer for strip development, obviating the need for a dedicated running buffer and reducing liquid handling steps. Through capillary action, the analyte is drawn up the strip and comes into contact with different antibodies along the way. In the 'sandwich' assay format—the most used type—the analyte interacts with the first monoclonal antibody at the conjugate pad. This antibody binds to an antigenic site on the analyte with high affinity, and is labelled with a reporter, commonly gold nanoparticles (AuNP). The antigen-antibody complex and unbound labelled antibody travel to the first detection line (test line) where another monoclonal antibody is embedded. This antibody targets a different epitope on the analyte, causing the latter to become sandwiched between two antibodies and yielding an intense purple band (colour of nanogold) at the test line. Excess AuNP-tagged antibody, on other hand, continues migrating to the second detection line (control line) and gets captured by the embedded antibody specific for the labelled antibody (Figure 8a). Thus, a positive sample generates two coloured bands on the strip, whereas a negative sample produces only one band at the control line. If the control band is not visible, the result is deemed invalid.

If two monoclonal antibodies to the analyte are not available, or if the analyte is too small to be bound by two antibodies simultaneously, the 'competitive' assay format can be employed. In this format, the test line is coated with the target analyte instead of an antibody. If the analyte is present in the sample, it sequesters the labelled antibody and prevents it from interacting with the embedded analyte at the test line. In contrast, when the target analyte is absent, the labelled antibody is free to bind to the embedded analyte. Consequently, in this format, a positive result is represented by

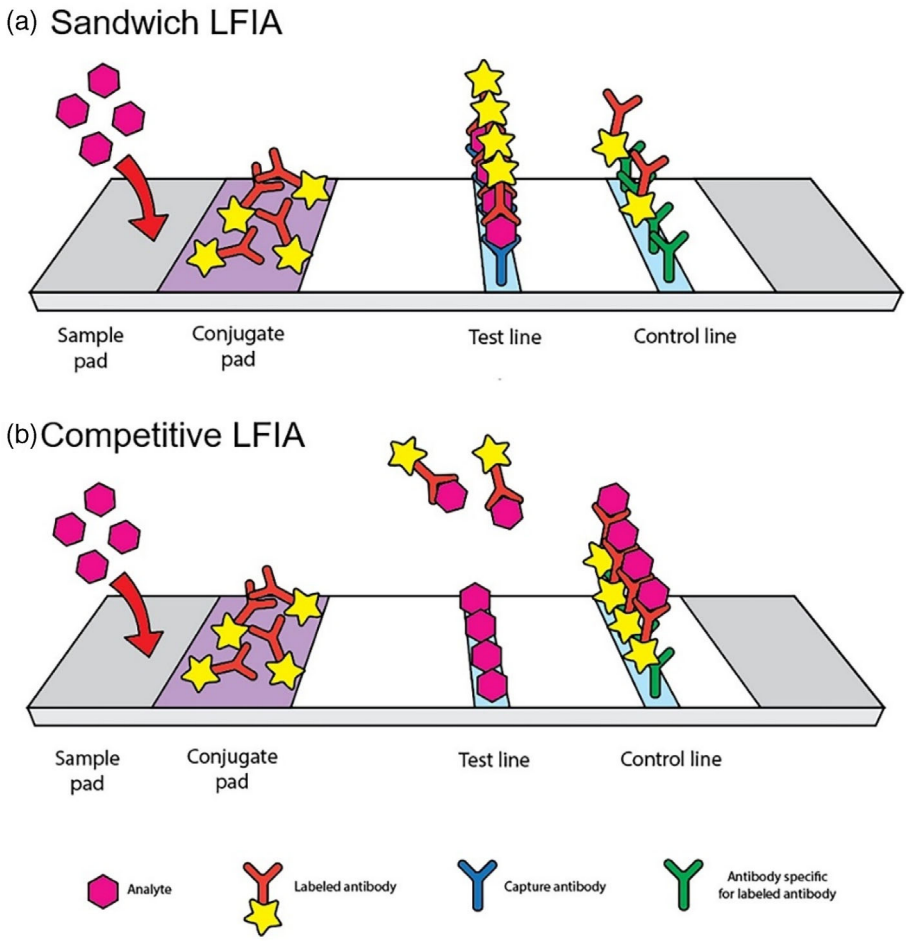

FIGURE 8 Schematic showing the composition and mechanism of sandwich (a) and competitive (b) LFIA. (Images by T. Chaijarasphong.)

TABLE 1 Pros and cons of available sequencing technologies

Technology	Read length	Total data	Pros	Cons
Illumina iSeq, MiniSeq, MiSeq and NextSeq, Novaseq*	2 × 150 bp 2 × 250 bp*	1.2–6000 Gbp	Becoming 'standard' for short reads. Accurate data, random error can be polished out. Established and well-validated, open source/community data analysis tools	Short reads High capital cost Requirement for laboratory infrastructure even for 'benchtop' units
Ion Torrent Personal Genome Machine (Thermo Fisher)	200 or 400 bp	30 Mbp to 2 Gbp	Fast output (2.3–7.3 h) Moderately priced	Short reads Limited community analysis tools Requirement for lab infrastructure
Pacbio Sequel II, Sequel IIe	30–40 kbp	160 Gbp per SMRT cell	Long reads, low systematic error rate (~0.1% for HiFi reads)	High capital cost Large footprint Requirement for lab infrastructure High run cost
Oxford Nanopore Technologies MinION	Up to 2.3 Mbp[164]	~30 Gbp per MinION flow cell (~10 Gbp per cell per day)	Long reads Low cost Pocket sized instrument No requirement for lab infrastructure or mains power Consensus error rate <0.005% (R10.4 flow cell) Open source/community data analysis tools	Systematic error rate ~5% for raw reads

Note: Data from manufacturers' websites, October 2021.

a single band at the control line while a negative result yields two bands on the strip (Figure 8b).

To date, LFIA tests have been developed for detection of diseases in a variety of fish species,[140–142] but so far only two are for tilapia pathogens, *Streptococcus agalactiae* and *Edwardsiella tarda*.[140,143] Although some pathogens, such as TiLV and *Flavobacterium columnare*, lack dedicated LFIA, effective antibodies against them have been identified and utilized to develop other immunoassays such as immunohistochemistry, enzyme-linked immunosorbent assay (ELISA) and fluorescence microscopy.[144,145] In addition, antibodies capable of recognizing host antibody directed against a specific pathogen have been identified, which may be useful for interrogating the present and past infection statuses of a fish population.[60,146] While these antibodies may serve as a good starting point for future development of LFIA, further optimization may be required, as an antibody that performs well in one type of assay may not perform well in another, due to differences in antibody affinity and concentration, chemical modification and microenvironment.

Although LFIA tests show great promise for routine disease diagnosis in the tilapia farming industry, there are still some issues that require attention. Currently available for only two tilapia pathogens, the cost of lateral flow strips constitutes a large fraction of the LFIA price per assay. Multiplex LFIA, capable of testing several pathogens at once, will significantly reduce cost. With greater utility and economic viability of the technology, LFIA should become more accessible and of greater use to tilapia farmers for disease diagnosis, improving protection from delayed detection or misdiagnosis of disease outbreaks.

3.4 | Next generation sequencing for fish disease diagnosis and epidemiology

Next generation sequencing (NGS) targeting molecular information from infectious organisms for diagnostic purposes has a long history, with the majority of standard methods for determination of infection status in humans, animals and plants now dependent on thoroughly validated PCR tests. These methods target highly specific loci of differentiation within the target pathogen, but provide little information beyond a well-defined case-positive or -negative within specified detection limits. Whole genome sequencing (WGS), on the other hand, provides the total information encoded in the genome of the pathogen, which contains a wealth of clinically relevant data; from antimicrobial susceptibility[147] to high resolution strain identity, that is valuable for epidemiology assessment and related disease control.[148–151] The value of such epidemiological detail has been highlighted through the global severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, where genomic information, provided in near real-time, was employed to identify case origins, and define quarantine controls which, in some cases, prevented further spread.[149,152,153] Indeed, epidemiological use of genomic data has attained global awareness as a result of daily updates from public health authorities.

In addition to targeted genome sequencing, NGS technology also lends itself to non-targeted or metagenomic NGS (mNGS), where total nucleic acid from a sample is sequenced directly, or generic regions such as 16S ribosomal RNA (16S rRNA) are amplified and then sequenced.[154] The resulting pool of sequence data can be de-noised,

assembled and analysed for presence and prevalence of possible pathogens. The non-targeted nature of mNGS makes it particularly useful for pathogen discovery. Indeed, the complete genome sequence of TiLV was first identified from metagenomic data derived from Illumina sequencing,[52] while a novel tilapia parvovirus HMU-HKU-1 was also discovered by Illumina sequencing from metagenomic libraries enriched for viral nucleic acids.[155]

While the advantages of genomic and metagenomic information to disease diagnostics and epidemiology are evident, they are relatively recent additions to the clinician's toolkit, largely due to the cost and time required to generate the information. Sequencing costs have fallen dramatically in the last two decades, greatly out-pacing Moore's law.[156] For example, it is estimated that the first human genome cost in excess of $100 million US. In contrast, the sequencing cost for the whole human exome (about 6 giga base pairs [Gbp] of data) is now around $1500 US. Sequencing cost is somewhat proportional to the amount of data required. Bacterial genomes are 1000 times smaller than a human exome at 2–6 megabase pairs (Mbp), and viral genomes even smaller, just 2–3 kilobase pairs (kbp) for nodaviruses, and 9–10 kb for TiLV.[157] Consequently, with adequate multiplexing, it is possible to generate sequence data for a bacterial genome for substantially less than $100 US. However, sequencing is only one part of the cost, so there is a strong tendency to underestimate the true cost of generating useful clinical genomic information, including sample preparation and downstream bioinformatics analysis.[158]

Current NGS technologies can be separated into two paradigms: (1) short-read and (2) long read sequencing.[159] There are pros and cons to each of the technologies and instruments currently in general use (Table 1). Short read sequencing is now dominated by the Illumina platform with very well-established laboratory preparation protocols and a wide range of well tested, open-source, data analysis tools and complete pipelines for mapping and assembly.[160] Moreover, there are excellent open-source tools for variant calling and clinically relevant typing, much of which can be performed directly from Illumina read data without need for time consuming assembly.[161,162] Short reads become problematic when structural elements need to be correctly resolved.[163] These might include critical plasmids, transposons or structures of long variant regions such as lipopolysaccharide (LPS) O-antigen and capsular polysaccharide (CPS) where rearrangement can lead to clinically relevant serotype switching. Pacbio Single Molecule Real-Time (SMRT) and Oxford Nanopore Technologies (ONT) nanopore sequencing are the major 'third generation sequencing' (TGS) platforms for generation of long read data that can fully resolve genomes to chromosome level. Because SMRT is polymerase-based, read-length is constrained by the enzyme chemistry and currently generates up to 30 kbp reads. SMRT provides high consensus accuracy due to effectively re-sequencing the same circular DNA constructs by the immobilised polymerase within the SMRT cell waveguide enabling highly accurate chromosome-level closure of genomes.[163] Nanopore directly sequences DNA molecules by actively drawing them through a biological pore in a solid state membrane while measuring the charge across the pore. The length of read generated is therefore only limited by the integrity of the DNA loaded, with the longest read recorded to date being 2,272,580 base pairs (bp).[164] The compromise with nanopore sequencing is relatively high systematic sequencing error (~5%) in raw reads, as the electrical resistance across the pore is influenced by several bases in the pore and their methylation state.[165] Nevertheless, the latest version of the nanopore MinION flow cells chemistry (R10.4), coupled with continuously improving base-calling algorithms, can provide a consensus accuracy of 99.995% from nanopore sequencing runs. The major advantage of the nanopore platform is the very low Minion instrument cost ($1000 US), and capability to operate the instrument under field conditions to generate clinical data in real time.[166]

3.5 | Application of WGS to fish disease diagnosis and epidemiology

In infectious disease investigation, genomic data is most useful for the high resolution it can deliver for epidemiology. The origins of disease introduction and most likely routes of transmission have been well-illustrated by WGS for some fish pathogens. For example, the trans-Atlantic dissemination of *Renibacterium salmoninarum* was postulated by genomic investigation,[167] while presence of serotype O2 *Yersinia ruckeri* in Tasmania and likely transmission of serotype O1b with salmonid eggs from Tasmania to Chile was also identified using NGS.[168] Introduction of piscine *Streptococcus agalactiae* serotype Ib into Australia, probably with imported tilapia in the 1970s and 1980s, and subsequent dissemination and evolution in wild marine fish populations was determined using Illumina short read sequencing.[169] However, NGS platforms have utility beyond WGS. Often, useful epidemiological and clinical information can be derived by sequencing amplicons generated by diagnostic PCR methods. For example, nanopore-based sequencing was recently employed to sequence diagnostic PCR amplicons for rapid genotyping of TiLV isolated from disease outbreaks in farmed tilapia.[170] The ability to conduct the sequencing locally and in near real time may be particularly advantageous in evidence-based outbreak control. Thus, a simple workflow for field application of nanopore sequencing in aquaculture may become a useful tool in the near future (Figure 9). In addition to simple field sample collection and processing protocols, utility of the technology will depend upon user-friendly interfaces that can interpret and correct read-data in real-time direct from the instrument and provide clinically relevant information back to the user, for example via a smartphone.

4 | WHAT IS ON THE HORIZON FOR EMERGING TECHNOLOGIES AND TILAPIA DISEASE DIAGNOSTICS?

4.1 | Artificial intelligent and machine learning

The rapid evolution of sequencing capabilities and costs, coupled to simplified analytical workflows, makes them accessible to fish disease

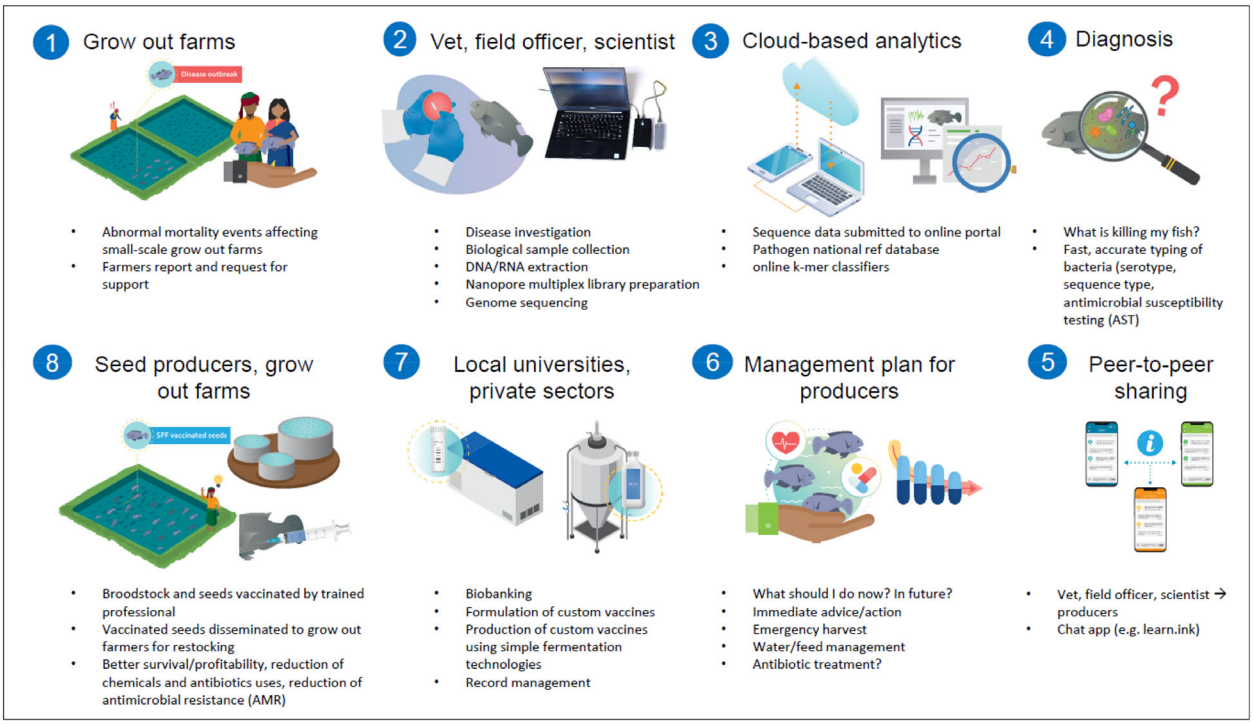

FIGURE 9 A hypothetical workflow for real-time field diagnostics using Oxford Nanopore sequencing. (Images by A. C. Barnes and J. Delamare-Debouteville.)

diagnostics with capacity to generate a mass of genomic data. Translating such data into clinical decisions or, at least, to information that is useful to clinical decision making by personnel on the ground, however, remains challenging. In the human genome context, the '$1000 genome and the $100,000 analysis' has been discussed.[171] Solutions, or partial solutions to this problem may lie in increasing use of artificial intelligence (AI). AI can be divided into expert systems and machine learning. Expert systems are devised around pre-defined sets of rules derived from clinical or veterinary experts to create a knowledge base that is mined by the expert system to provide computer-aided decision support.[172] However, as scenarios become more complex, as indeed they are in the diagnosis of infectious diseases in aquaculture environments, expert systems are clearly limited by the information in the knowledge-base. Machine learning overcomes this constraint by employing algorithms that devise and refine their own sets of rules from data, allowing them to learn as more data become available. Ensuring the quality of the training data then becomes the major limitation.[172] Machine learning is already integrated into the ONT' base calling algorithms for interpretation of the current signal into bases, with several available nanopore community and open source bioinformatics post-processing applications, all based on artificial neural networks.[165] To get from sequence to clinically relevant actionable information is more challenging. For example, predicting antimicrobial susceptibility to enable rapid evidence-based therapeutic intervention is feasible from whole genome or metagenomic data using neural networks.[173] Predicting antibiotic susceptibility direct from raw nanopore sequencing reads was an early application of the technology.[174] To provide comprehensive clinical and epidemiological information on infectious agent, serotype, sequence type and antimicrobial susceptibility, direct from sequence reads is feasible by taking a k-mer approach.[175] Although there is a high computational overhead to k-mer based analysis as datasets become large, by using an application-specific database (e.g., fish pathogens) and binning k-mers into differentially descriptive subsets,[175] a classifier based on this approach is highly feasible for fish infectious disease diagnostics. Indeed, there is an open access development release of a k-mer classifier and associated database for pathogens of aquatic organisms including tilapia available from WorldFish.[176] This is a field that is moving very rapidly and the choice of online tools that are easy and free to use is growing. The Danish Technical University provides a suite of online tools and databases through their Centre for Genomic Epidemiology portal including, for example, pathogen identity, antimicrobial resistant genes (ARG) prediction and multilocus sequence typing (MLST) direct from raw sequence data.[177,178]

Artificial Intelligence may also become applicable to Level I and Level II diagnostics through interpretation of real-time environmental and behavioural cues (level I) to alert to potential problems, although perhaps not to the level of specific disease diagnostics. Sensor arrays for water and environmental monitoring, measuring and controlling feed intake and in-tank/cage camera systems for morphometric analysis are already widely deployed throughout salmonid aquaculture for automation. Coupling to AI is therefore highly plausible to provide computer-assisted level I diagnostic alerts. For tilapia aquaculture, the costs of sensor infrastructure will need to fall substantially to enable adoption in the most important producing nations. Level II diagnostics

are already assisted by AI in human clinical medicine, particularly cancer diagnostics, where screening of histopathological samples may be aided by deep neural network-based machine learning algorithms.[179] It is also possible to combine AI with other more rapid Level II laboratory methods such as infra-red (IR) spectroscopy (30 min)[180] or flow cytometry (3h)[181] to provide same-day antimicrobial susceptibility test (AST) results and predict bacterial abundance. Indeed biomarkers from blood measured by IR-spectroscopy coupled to artificial neural networks can provide rapid non-invasive diagnosis of *Helicobacter pylori* infection in children.[182]

4.2 | High throughput diagnostic systems

For local diagnostic testing of fish farm disease outbreaks, high throughput of sample numbers is not a major factor, and high capacity instrumentation can be expensive to operate when not used at capacity. However, there is a use-case for high throughput diagnostics in pathogen surveillance for biosecurity. For example, the screening of broodstock and seedstock to certify specific pathogen free (SPF) status, or for the screening of live or uncooked seafood prior to international shipping for compliance with trade legislation to limit transboundary spread of endemic diseases. For advances in high sample number throughput for pathogen detection, we return to the SARS-CoV-2 pandemic. Here, rapid testing of hundreds of thousands of samples per day by health authorities and the private sector has informed lockdowns and tracked dissemination of new virus variants.[149,183] For aquaculture biosecurity, the need is somewhat different, in that testing fewer samples for a cohort of pathogens of concern is more important than testing high sample numbers for a single pathogen. But there are important advances made during the pandemic that can be applied equally well to fish disease diagnostics. For example, one of the major constraints (and costs) of diagnostics is in sample preparation with many recommended molecular assays stipulating particular extraction kits.[184] Recent findings indicate that for qPCR-detection of SAR-CoV-2 from clinical samples, the extraction process can be substituted for a short high temperature treatment without adversely impacting sensitivity.[184] Molecular assays lend themselves very well to high throughput as the small reaction volumes that are required facilitate use of microwell plates (e.g., 384 wells) and array type technologies. qPCR methods are standardised for many pathogens of fish and are readily multiplexed by using different fluorochromes in probe-based qPCR such as TaqMan. For tilapia, multiplexed qPCR detection of common bacterial pathogen, *Francisella* spp., *Edwardsiella* spp. and *Streptococcus* spp. was effectively used for disease surveillance on hatcheries in Costa Rica.[94] The extent to which assays can be multiplexed in this way is quite severely limited by the range of fluorochromes and the number of channels on the instrument that can detect the differing wavelength emissions. Once internal controls are accounted for, four to five pathogens per sample is the limit to which the assay can be multiplexed. This problem can be reduced by coupling the qPCR to electrospray mass spectrometry, in which the qPCR amplicons from the multiplexed primer reaction are fed to a mass spectrometer which then identifies which amplicons are present in each sample by mass, eliminating the need for fluorochrome probes.[185] This method may enable quantitative detection of 13 or 14 different pathogens per sample in a single reaction and is limited by the biochemistry of the qPCR reaction with higher numbers of multiplexed primer sets. For increased pathogen multiplexing, microarray-based chips may include thousands of genetic loci with potential to identify tens to hundreds of pathogens to variant level.[186] Such arrays are quite costly but have been used in human medicine for screening blood samples,[187] and DNA microarray genus-species 16S rRNA analysis for multiplexed detection of key pathogenic bacteria have been explored in aquaculture.[188] High throughput microarray methods for tilapia disease diagnosis are limited but may offer future perspectives to cover all key pathogens of tilapia including bacteria, viruses and parasites.

4.3 | Environmental DNA and RNA for early detection of pathogens from water

Environmental DNA and RNA (eDNA and eRNA) refer to genetic materials found in environments such as water, soil, sediment, snow or even the air. eDNA/eRNA include those within or shed and excreted from any living or dead organisms, from viruses to unicellular and multicellular organisms.[189] Sample collection for eDNA/eRNA investigation can be done once, or on a regular basis at a certain timeframe and location for continuous monitoring. Following that, the samples are treated to appropriate concentration processes (commonly filtration, centrifugation or coagulation) before DNA, RNA or total nucleic acid are extracted[190,191] (Figure 10). The obtained eDNA/eRNA is then subjected to either a metagenomic NGS (or metabarcoding) approach, in which the contribution of organism taxa can be identified simultaneously, primarily at the genus level, or a target-specific conventional or quantitative PCR for detection of species of interest[189,192,193] (Figure 10). Application of eDNA/eRNA has played an increasingly important role in both common and unusual circumstances in aquatic ecosystems and aquaculture. Monitoring eDNA, for example, can be used to look at organism diversity in the context of natural conservation or to assess the biological impact of climate change, changes in environmental parameters and anthropogenic activities (e.g., oil spill, drilling and mining).[194,195] eDNA/eRNA can be applied for disease screening to ensure free status of any pathogens of concern particularly for biosecurity in the fish/shrimp trade.[196] eDNA monitoring can help identify invasive species and assess endangered species in aquatic habitats.[197–199] Furthermore, eDNA/eRNA has been used to assess the distribution and abundances of waterborne pathogens, as well as the presence of pathogenic agents in the environment.[191,200]

The application of eDNA/eRNA for tilapia disease diagnosis is still limited, however, a straightforward approach for TiLV detection and quantification from water that employed a simple iron flocculation method for viral concentration coupled with a probe-based RT-qPCR has been described.[55] TiLV nucleic acid was detected and quantified in water collected from affected ponds/cages as well as sewage, and a reservoir. This approach might be effective for noninvasive monitoring of TiLV in aquaculture environments, and allow suitable biosecurity

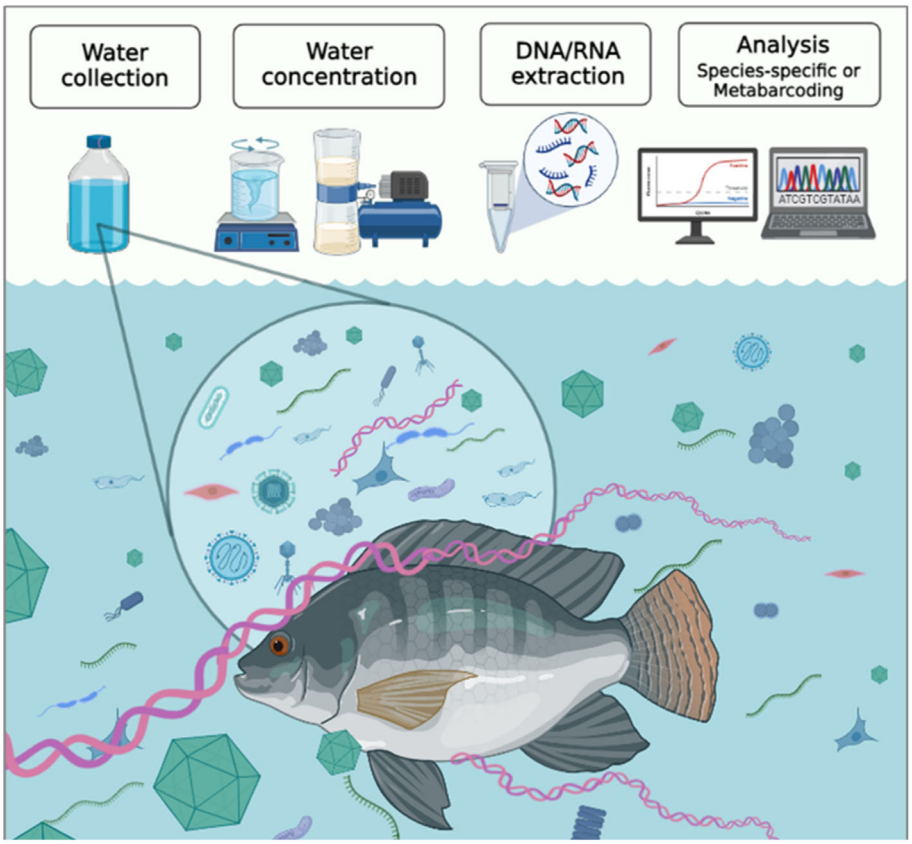

FIGURE 10 eDNA/eRNA application in tilapia disease diagnosis. Pathogen(s) collected with water samples from fish culture systems are usually concentrated prior to nucleic acid extraction. Pathogen(s) of concern can be detected by species-specific or metabarcoding approaches. (Images by S. Senapin and S. Taengphu created in BioRender.com.)

interventions.[201] Potential applications of eDNA/eRNA in disease diagnosis have also been described in other fish species and their pathogens, including the use of pathogen-specific detection approaches, metabarcoding strategies and a combination of both. For instance, the detection and quantification of red sea bream iridovirus (RSIV) in a challenge model with Japanese amberjack (*Seriola quinqueradiata*) and farmed red sea bream (*Pagrus major*) revealed high viral loads at least 5 days before fish mortality, suggesting potential application of eDNA assay for early forecast of disease.[55,202,203] Multiple target pathogens were detected using eDNA samples collected from Atlantic salmon (*Salmo salar*) farm sites to assess the potential of pathogen transmission from domesticated to wild fish populations sharing the same habitat.[204] The use of universal metabarcoding markers (e.g., mitochondrial genes, internal transcribed spacer (ITS) sequences and small-subunit ribosomal RNA gene) as potential monitoring tools for harmful parasites and microalgae in cultured fish have been described.[205] A synergistic association of bacterial microbiome and abundance of the parasitic ciliate *Chilodonella hexasticha* with mortality in barramundi (*Lates calcarifer*) has been demonstrated using a combination of metabarcoding- and targeting-based approaches.[206] eDNA assays, on the other hand, have indicated an antagonistic effect between bacterial loads and viral pathogens.[207,208] As aquaculture is an interactive complex system, environmental parameters together with host and pathogen factors should be taken into account for eDNA/eRNA data analysis and interpretation. The advancement of technology in the eDNA/eRNA methods described in other fish species can easily be used for tilapia health monitoring and disease diagnosis. Availability of curated genomic sequence databases of tilapia pathogens and other aquatic organisms characterized from healthy and diseased tilapia culturing environments will support accurate eDNA/eRNA species-level identification and interpretation of complex microbial assemblages. In the near future, more accessible and inexpensive NGS and qPCR/dPCR facilities and services will promote a rise in the use of eDNA/eRNA for early diagnoses and disease forecasting in tilapia farming systems.

4.4 | Point-of-care or pond-side testing

The term 'point-of-care testing' (POCT) describes diagnostic tests, or any other tests, that are not confined to a laboratory setting and, thus, can be conducted close to/in the direct proximity of the testing subjects, typically by people without professional training. Different circumstances may require different POCT solutions involving different testing devices or regimes. For fish farmers, POCT allows

anyone to easily and quickly perform accurate testing close to or at the pond side. POCT may also be undertaken in many locations such as fish processing plants, wet-markets or by customs biosecurity officers for monitoring and screening purposes. In summary, its relevance in aquaculture may include 'pond-side testing, 'point-of-need testing', 'remote rapid testing' or 'decentralized testing'.[209,210]

Accurate diagnostics for effective treatments are not available for many infectious diseases in tilapia, making good farm practices and prevention the best strategies for achieving optimum performance results. A rapid, accurate and reliable diagnosis allows the farmers to make immediate and informed decisions and take appropriate actions in the fastest manner possible to better manage and control diseases, especially at early stages when clinical signs may not be easily identified by the farmers.[211] However, most tilapia farms exist in relatively remote locations with limited accessibility to laboratory testing facilities. Sending clinical samples to specialized laboratories has the drawback that it usually takes a long time (days to weeks) to obtain test results. For diseases that quickly lead to high morbidity and/or mortality, having results one or two weeks after sample submission is not optimal. Therefore, POCT tools that provide quick and reliable testing results at the tilapia farm level are much needed to shorten the test turn-around time for timely decision-making.[14]

An ideal POCT should meet the 'ASSURED' guidelines (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Delivered)[212] put forward by the WHO.[213] POCT should provide test sensitivity and specificity comparable to those of laboratory testing in a short time under a wide range of conditions. The equipment, when needed, should be compact, work with a simple operating protocol, with battery and built-in calibration and provide means for data management (such as test results, date, time, sample, operator, location, quality control and device info).[214] Ideally, the reagent should be provided in single-dose and ready-to-use format and require no cold chain for shipping and storage. Concerns about POCT focus mainly on risk of poor test performance due to oversights, such as potential user errors, insufficient quality control and inadequate storage of reagents and maintenance of devices (if any). Therefore, clear instruction guides, user training, user-friendly design and usability validation are some measures known to ensure correct use of POCT.

Lateral flow immunoassay (LFIA) is the most widely commercially available POCT platform. Working with little or no supporting infrastructure, LFIA has advantages of being simple, rapid and cost-effective. These features are very useful, especially in settings with low resources, to improve decision making and turn-around time. LFIA can be used to screen for infection and antibiotic resistant markers to facilitate responsible and prudent use of antimicrobial agents.[215–217] However, to the best of our knowledge, no LFIA tests for infectious diseases of tilapia are commercially available at time of print.

Current LFIA tests are, in general, not consistently sensitive and specific enough to meet the needs of early disease detection, especially sub-clinical infections.[218] Therefore, tests based on molecular technologies are considered more reliable with greater sensitivity for this purpose. Laboratory molecular technologies mentioned in the previous sections, such as PCR, LAMP, RPA, CRISPR and the Nanopore MinION sequencing platform, have all been automated into single use commercial POCT assays prepacked with required reagents for diagnosis of COVID-19 infection.[219] The automated steps include sample preparation, nucleic acid extraction, amplification, signal detection, recording and processing and result interpretation and presentation. Besides being able to improve test specificity with its ability for strain identification with single-nucleotide specificity through CRISPR base-pair matching, CRISPR-based diagnostics for pathogen detection also hold great promise in facilitating equipment-free diagnostics to allow POCT to be easily accessible to more users.[220,221] However, the majority of available CRISPR-based platforms require an amplification step to enhance sensitivity, significantly lengthening test turn-around time.[222] Although a number of PCR, LAMP and RPA assays have been reported for detection of TiLV, ISKNV, *S. agalactiae*, *S. iniae*, *L. garvieae* and *F. columnare*,[67,105,107,223] to the best of our knowledge, only one POCT RT-PCR test that works on the compact POCKIT platform (GeneReach, USA) is commercially available for TiLV detection in tilapia. Designed to work with the fluorescence-based insulated isothermal PCR (iiPCR) technology,[224,225] this compact platform provides fast binary (positive/negative) results. Based on this platform, semi-automated (POCKIT Combo) and fully automated (POCKIT Central) systems are available for pond-side PCR testing at different settings. The semi-automated POCT system generates results within two hours with a protocol requiring minimal manual steps; one nucleic acid extract can be used flexibly for simultaneous PCR testing of different pathogens. The sample-in-answer-out POCT system, on the other hand, fully automates the nucleic acid extraction and iiPCR steps and works with preloaded single-use cartridges to provide results within 90 min, meeting particularly the needs of settings with limited human resources.

The TiLV POCT RT-iiPCR assay is available in a lyophilized format for easy shipping and storage. On the fully automated POCKIT Central system, LoD 95% (limit of detection) of the POCT assay was determined to be 12 genome equivalents. The POCT assay was comparable to a reference semi-nested RT-PCR assay[47] in analytical and clinical performance. The two RT-PCR assays have similar analytical sensitivity as their detection end points were within one log in a test using a serial dilution of a TiLV-positive sample. A study testing 92 tilapia liver, brain, gill, muscle or mixed samples showed that diagnostic performance of the two assays was also comparable. Positive percentage agreement and negative percentage agreement were 94.44% (95% CI, 78.72%–100%) and 95.95% (95% CI, 90.4%–100%), respectively (GeneReach Biotechnology Corporation data).

Development of commercial LFIA tests for the tilapia industry may consider incorporating dedicated LFI readers and alternative detection methods (fluorescence, chemiluminescence, electrochemical signals, surface-enhanced Raman spectroscopy).[216,226] These technologies have potential to improve test sensitivity and enable

quantitative testing. The use of readers also makes data digitalization, tracking, storage and transmission possible.

The functions of POCT are being improved to enhance its usability for different applications at various point-of-care settings. First, the ability of multiplexing is favourable to improve testing efficiency of POCT.[227,228] Second, integration of easy, or no, sample preparation enhances user-friendliness of POCT.[229] Thirdly, miniaturized integrated devices are being developed to enhance test portability and user-friendliness.[230] In the last decade, huge progress has been made in microfluidics and microfabrication technologies that enable automated pipetting, mixing, separation and amplification in a single miniaturized device, with significant reduction in sample and reagent volume, test turn-around time, energy consumption and waste production.[230] Fourthly, improving connectivity of POCT to allow integration of accurate outbreak reporting systems via a mobile app or computer connections, can help with timely and accurate reporting of outbreaks to competent authorities.[214] Moreover, cloud-based reporting and artificial intelligence (AI) have potential to further bridge what scientists and aquatic health professionals can offer to meet the needs of tilapia farmers at remote locations.[231] The momentum accumulated in the last decade in amplification, multiplexing, microfluidics and data connectivity technologies, could be integrated realistically and in different ways to build cost-effective POCT for the tilapia aquaculture industry in the near future.

Continuous research and enhancement of POCT with the goal of providing end-users with better and simpler access to biodetection techniques will assist farmers in disease management and control enhancing future tilapia productivity. Currently available techniques are not widely used in aquaculture settings, owing mostly to their relatively expensive prices, thus, efforts are also required to reduce the costs of POCT.

5 | CONCLUSION

In aquaculture, diagnostic techniques are constantly evolving and becoming more complex. The level I-III approach established over 20 years ago highlights the importance of the diagnostic continuum as a quality control mechanism, especially for exotic or previously unreported mortality events. They remain meaningful in light of diagnostic technology advances and increasing recognition of the role of the aquatic environment on both host physiology and pathogen virulence. Accurate diagnosis of a disease can rarely be achieved by a single test. A presumptive diagnosis, indicating a strong likelihood of disease identification, is usually made with multiple tests to be considered for confirmatory diagnosis (100% certainty of identification of the causative pathogen). In order to reduce the risk of misdiagnosis, inclusion of three levels of diagnostic observations and use of a matrix of results gives the most solid foundation possible for accurate diagnosis. This is essential for effective risk assessments at the farm, regional, national and international levels of aquaculture production, as well as for effective disease response and control. Accurate diagnosis forms the basis for determining what the disease condition is, the severity and cause(s) of the condition. Inaccurate diagnoses can lead to ineffective or inappropriate control measures, delay treatment and may cause severe economic loss. The choice of diagnostic technique should follow the principles of being 'fit-for-use, fit-for-purpose' with defined sensitivity and specificity and cost-effectiveness within the pathogen–host–aquatic environmental interaction framework. Diagnostic challenges to detect 'unknowns' and 'emerging diseases' will persist, however, our increasing molecular databases and analytical tools should enhance our capability to detect and identify these new pathogenic agents more rapidly and accurately in the future compared with the present.

The intrinsic qualities of tilapia, as well as its biology, farming needs and nutritional values, give it the inherent potential to become one of the world's most important future food fish groups. The inter-relationship of human, animal and environmental health enshrined in the One Health philosophy, that is beginning to underpin global health policy, means that the future of tilapia aquaculture must centre on sustainable health management and biosecurity. There has been a rapid proliferation in the development of novel diagnostic methods, with many technical challenges having been overcome. The major hurdle that faces the adoption of such powerful aids to diagnosis is likely to be the rigorous validation required for them to be accepted for transboundary animal movement and product entry into supply chains. We recognize the potential for misapplication of new technologies in aquaculture disease diagnostics, including tilapia, in the absence of other diagnostic information and we emphasize the importance of three continuous levels of disease diagnostics that incorporate fundamental (Level I and II) and advanced (Level III) approaches to optimize the diagnostic data value. It is likely that LAMP, and NGS methods for tilapia pathogens will be validated and join WOAH standard diagnostic tests, such as qPCR, in the near future. We also expect to see incorporation of artificial intelligence, machine learning, high throughput diagnostic systems and POCT into diagnostic workflows in the relatively near future. Non-invasive sampling using eDNA, in conjunction with highly sensitive diagnostic technologies such as qPCR and dPCR for early pathogen detection and disease forecast, should also be incorporated in the coming years. Regulatory and socio-economic hurdles aside, the technology for fast, easy, accurate and farmer-accessible diagnostic tools for future sustainable aquatic food is already here.

AUTHOR CONTRIBUTIONS

Ha Thanh Dong: conceptualization; investigation; methodology; writing – original draft; writing – review and editing. **Thawatchai Charijarasphong:** Formal analysis; investigation; methodology; visualization; writing – original draft. **Andrew Barnes:** Investigation; methodology; visualization; writing – original draft; writing – review and editing. **Jerome Delamare-Deboutteville:** Investigation; methodology; visualization; writing – original draft; writing – review and editing. **Peiyu Alison Lee:** Investigation; methodology; writing – original draft. **Saengchan Senapin:** Investigation; methodology; visualization; writing – original draft. **Kathy F. J. Tang:** Conceptualization;

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CONFLICT OF INTEREST

The views expressed in this publication are those of the author(s) and do not necessarily reflect the views or policies of the Food and Agriculture Organization of the United Nations.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created in this review.

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