

Potential Application of PCR Based Molecular Methods in Fish Pathogen Identification: A Review

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Abstract

Molecular biology developments have led to fast growth in new methods for fish disease diagnosis. Molecular diagnostic methods are rapid and more specific, more sensitive than the culture of pathogens, serology, histology, and biochemical methods which are traditionally utilized to identify causative agents of fish disease. Molecular diagnostic methods are valuable for detecting specific pathogen that are difficult to culture *in vitro* or require a long cultivation period and it significantly more rapid in providing results compared to culture. It enables earlier informed decision-making and rapid diagnosis of bacteremia, particularly for low levels of bacteria in specimens. Molecular techniques which have the major significance are mainly PCR-based molecular diagnostic methods including Polymerase Chain Reaction (PCR), Real-Time Polymerase Chain Reaction (RT-PCR), Multiplex Polymerase Chain Reaction (multiplex-PCR), and Random Amplified Polymorphic DNA (RAPD), etc. These have been increasingly utilized to diagnose fish disease for the last recent years. Molecular diagnostic methods can detect pathogens from asymptomatic fish, so disease outbreaks could be prevented. As a consequence, antibiotic treatment can be reduced and the development of antibiotic-resistant bacteria can be eliminated. In this review paper, we attempt to summarize the potentiality of PCR-based molecular diagnostic methods and their application in fish pathogen identification.

Introduction

Aquaculture industries are major contributors to the economy of Bangladesh (Ahmed, 2013; Dey *et al.*, 2008) as well as many countries and an increasingly important component in global food supply (Charoonnart *et al.*, 2018). The fast expansion of aquaculture has been accompanied by the spread of infectious diseases and accountable for vital economic losses (Leung & Bates, 2013; Tavares-Dias & Martins, 2017). In the attempt to combat infectious diseases,

biocides and antimicrobials are used worldwide, with subsequent hazardous outcomes to the environment and workers' health, and the increased chance of the emergence of resistant strains (Cabello, 2006; Defoirdt *et al.*, 2011;). The fish diseases are a greater problem in the sustainable development of Fish culture (Bondad-Reantaso *et al.*, 2005) in Bangladesh as well as in the world (Rodger, 2016). Bacteria and virus are the leading causative agents of diseases (Plumb & Hanson, 2010; Wolf, 2019) in freshwater fishes (Pridgeon & Klesius, 2012) and marine fishes (Muroga, 2001).

Fish disease diagnosis has progressed from the traditional approaches of isolation and phenotypic characterization of the pathogen to the modern methods of molecular biology (Austin, 2019). Traditionally, the diagnosis of the disease is operated by agar cultivation and then observation of phenotypic and serological properties of the pathogen and sometimes histological examination (Gilligan, 2013; Kumar *et al.*, 2014). In many developing countries, fish pathogens are usually identified based on traditional biochemical identification methods (Adhikari *et al.*, 2015; Váradi *et al.*, 2017) which are time-consuming and most regularly could no longer confirm the pathogen specifically (Foddai & Grant, 2020; Franco-Duarte *et al.*, 2019). Some experiments have been made using biochemical tests, DNA homology, and protease variability (Kumar *et al.*, 2014; Zdzalik *et al.*, 2013), these strategies have some negative aspects such as the need for previous isolation of the pathogen and inadequate sensitivity to detect low levels of the pathogen (Altinok *et al.*, 2008; Wakabayashi *et al.*, 2016).

In contrast, molecular identification techniques are very robust and precise (Adzitey *et al.*, 2013; Franco-Duarte *et al.*, 2019) and currently practiced in different developed (Bigarré *et al.*, 2017) and developing countries (Sarowar *et al.*, 2019). During the last fifteen years or so, molecular methods have been increasingly more utilized to diagnose fish diseases (Altinok & Kurt, 2003). Since molecular diagnostic strategies are quicker and greater sensitive (Dwivedi *et al.*, 2017; Kociolek, 2017) than traditional diagnostic techniques, pathogens could be identified from asymptomatic fish resulting in prevention of diseases before an outbreaks.

Fish health management is a term utilized in aquaculture to describe management practices that are planned to avoid fish infection (Assefa & Abunna, 2018; Opiyo *et al.*, 2018). Once fish get in poor health it can be challenging to salvage them. Successful fish health management starts with the prevention of disease as an alternative to treatment (Francis-Floyd, 2011; Noga, 2010). Under intensive aquaculture conditions, the risk of stress increases, and a massive proportion of the stock might also come to be infected (Datta, 2012; Huntingford & Kadri, 2014; Rehman *et al.*, 2017). Therefore, the detection of the pathogen from carrier fish is necessary for effective fish disease management (Sadler & Goodwin, 2007). In this aspect, the main objectives of this review are to explore the potentiality of six PCR based molecular diagnostic methods: i. PCR; ii. Multiplex-PCR; iii. RT-PCR; and iv. RAPD applied for fish pathogen identification v. Nested PCR vi Loop-mediated isothermal amplification (LAMP).

Glance of Molecular Diagnostic Methods

Diagnosis is the process of identifying the disease or any abnormal condition which is explain and derived from the host's sign and symptoms. The data required for diagnosis is normally derived from a history and

physical test of the host organism seeking care (Abdisa & Abdisa, 2017; Neshati *et al.*, 2018), sometimes, one or more diagnostic procedures like clinical tests, are also accomplished during the procedure (Balogh *et al.*, 2015). According to Aggarwal (2015) "Diagnosis may be defined as the determination or identification of the cause or nature of an illness by evaluation and analysis of the signs, symptoms and supportive tests in an individual patient. Diagnostic criteria are a set of signs, symptoms, and tests for use in routine clinical care to guide the care of individual patients" (Aggarwal *et al.*, 2015). According to Patrinos *et al.* (2017) "Molecular diagnostics is referred to as the detection of genomic variants, aiming to facilitate diagnosis, sub-classification, prognosis, and monitoring response to therapy". Molecular diagnostics is the compiled result of fruitful interaction amongst laboratory medicine, genomics, and technological knowledge in the area of molecular genetics, in particular with significant discoveries in the subject of molecular genomic technologies (Dwivedi *et al.*, 2017;). All these factors make a contribution to the identification and high-quality characterization of the genetic foundation of the pathogen which, in turn, is fundamental for the accurate provision of diagnosis (Dwivedi *et al.*, 2017). Molecular diagnostics is a series of methods used to analyze markers in the genome and proteome the individual's genetic code and how their cells express their genes as proteins by way of applying molecular biology to clinical testing (Orakpoghenor & Markus, 2020; Shen, 2019). Molecular techniques can keep away from problems inherent in the study of organisms for which no *in-vitro* culture medium or technique is available, and have the possibility to significantly increase the sensitivity of detection (Francy *et al.*, 2009; Rhoads *et al.*, 2012). Many molecular techniques for diagnosis are available to detect the genetic variation among pathogen genes (Amjad, 2020; Procop, 2007). Such as Polymerase Chain Reaction (PCR) based are: Real-Time Polymerase Chain Reaction (RT-PCR) (Logan *et al.*, 2009; Wong & Medrano, 2005), Multiplex Polymerase Chain Reaction (multiplex-PCR) (Mahoney & Chernesky, 1995;), Random Amplified Polymorphic DNA (RAPD) (Butler, 2012) etc. , and non-PCR based are: Restriction fragment length polymorphism (RFLP) (Mittal *et al.*, 2013), the enzyme-linked immunosorbent assay (ELISA)(Drijvers *et al.*, 2017), etc.

The Potential Beneficial Role of Molecular Diagnostic Methods in Aquaculture

Successful fish health management starts with the prevention of disease as an alternative to treatment(Faisal *et al.*, 2017). Under intensive aquaculture conditions, the risk of disease outbreak, anti-biotic resistant bacteria, aquatic pollution, stock mortality etc. are common phenomena (Preena *et al.*, 2020; Santos & Ramos, 2018). Once fish get in poor health it is very challenging to manage them (Assefa &

Abunna, 2018). Therefore, the detection of pathogen from the host is necessary for effective fish disease management (Altinok & Kurt, 2003; Austin, 2019).

Prevention of Disease Outbreak

The molecular method for disease detection is now a world-recognized system (Dwivedi *et al.*, 2017). The fish pathogen can be detected accurately with the help of molecular diagnostic methods (Cai *et al.*, 2014). In case of traditional biochemical tests, many fish pathogens cannot be detected even closely related pathogenic species cannot be differentiated (Adhikari *et al.*, 2015; Bajinka, 2017). In most cases of aquaculture, accurate identification of causative agents of diseases is not practiced, so it is quite difficult to apply the proper management for preventing and using appropriate drugs for controlling the diseases (Assefa & Abunna, 2018). As consequence, an outbreak of diseases occurred frequently (Lindahl & Grace, 2015; Sharma *et al.*, 2012). If pathogens can be detected and recognized in the environment, for example, between harvesting and re-stocking, then this can be extremely useful in the prevention of disease outbreaks (Assefa & Abunna, 2018; Dwivedi *et al.*, 2017).

Reducing the Chance of Developing Antibiotic-Resistant Bacteria

Antibiotic-resistant bacteria is a burning issue in Aquaculture. The capacity of Antimicrobial resistance (AMR) genes to pass between microbes, a technique known as horizontal gene transfer (HGT), is thought to underlie the quick rise in safe pathogens seen over the globe. (Thorner *et al.*, 2020; Watts *et al.*, 2017). This process can happen between irrelevant bacterial species, meaning that resistance genes display in non-pathogenic, natural microbes can be exchanged to the creature or human pathogenic microbes and posture a danger to the creature and human wellbeing (Fletcher, 2015; Peterson & Kaur, 2018; Thorner *et al.*, 2020). Accurate diagnosis of disease allows for the selection of appropriate antimicrobials, and avoidance of antibiotics in human health (Abadi *et al.*, 2019; Llor & Bjerrum, 2014). Therefore, antibiotic treatment can be reduced and the development of antibiotic-resistant bacteria may also be eliminated.

Other Beneficial Role of Molecular Diagnostic Methods

Molecular methodologies offer numerous advantages to the clinical laboratory. These include:

Turnaround Time

In comparison with standard conventional culture strategies, molecular techniques ordinarily offer way better turnaround times from receipt to result announcing (Leung & Bates, 2013; Lievens *et al.*, 2011).

Application Area

Broader applications can be found with molecular methodologies such as infectious diseases, genetic testing, drug resistance, and tumor marker detection etc. (samples collected from fish tissue, blood, etc.) (Debnath *et al.*, 2010; Emmadi *et al.*, 2011; Marwal & Gaur, 2020).

PCR Based Methods in Fish Pathogen Identification

Polymerase Chain Reaction (PCR)

In 1983, the Polymerase Chain Reaction (PCR) was discovered by Kary Mullis (Mullis *et al.*, 1986). PCR resembles an in vitro and elementary form of DNA replication, a physiological process used by all living cells to duplicate their genetic material before cell division (Baynes & Dominiczak, 2009).

Denaturation

DNA Template contains the sequences that will be amplified by PCR (Kalle *et al.*, 2014). In this stage, melting temperature (T_m) generally 92–94°C makes the double-stranded DNA separate into the single-stranded (Borah, 2011; Sarah Maddocks & Jenkins, 2017).

Annealing

The short DNA fragments are called “Primers” that bind by complementary base pairing to opposite DNA strands at the annealing temperature generally 55–60°C (Borah, 2011; Tymoczko *et al.*, 2011).

Elongation

DNA polymerases copy DNA molecules during the PCR reaction (Caetano-Anollés, 2013; Drouin *et al.*, 2007). DNA polymerase is an enzyme responsible for DNA replication and only able to add nucleotides to the 5′ to 3′ end (Cox *et al.*, 2015).

Theoretically, the increase in the amount of product after each round will be geometric (Rimstad *et al.*, 1990). In every cycle of PCR, approximately the amount of DNA increased to double, as well as it is repeating in the following cycle and, a new strand of DNA subsequently acts as a template for replication. This results in an exponential increase in the number of targeted segments of DNA during PCR. A total of 25–40 PCR cycles is carried out and depending on the expected yield of the PCR products (Baynes & Dominiczak, 2009). Usually, at least a millions copy of a particular region of a DNA molecule can be produced and the PCR product can be detected via gel electrophoresis (McPhearson *et al.*, 1991). The amplified product may then be used for analytical detection, sizing, cloning, or sequencing (Wages, 2005).

Major Advantages of PCR

PCR method is valuable for detecting specific pathogens that are difficult to culture in vitro or require a long cultivation period (Yamamoto, 2002) and it is significantly more rapid in providing results compared to culture. It enables earlier informed decision-making by the rapid diagnosis of bacteremia, particularly for low levels of bacteria in specimens (Maurer *et al.*, 2017). It is useful in detecting cases in extrapulmonary specimens which may be missed by smear and/or culture (Narayana *et al.*, 2018). PCR is still considered an adjunct test for certain diagnostic tests that still rely on smear and culture (Yang & Rothman, 2004).

Disadvantages of PCR

PCR still need culture for testing for drug/antibiotic susceptibility and genetic typing (Cockerill, 1999). PCR results should not be used as the sole basis of a patient treatment management decision. All results should be interpreted by a trained professional in conjunction with the review of the patient's history and clinical signs and symptoms. False-negative results can arise from improper sample collection/transport, an insufficient amount of specimen, degradation of nucleic acids (typically RNA) during shipping or storage, detecting organisms representative of normal flora near specimen collection site, acid-fast bacilli in water, and contaminants in the lab, specimen mix-up, etc (Narayana *et al.*, 2018).

Application of PCR to Identify Fish Pathogens

A lot of Polymerase chain reaction (PCR) assays are already developed to identify the specific fish pathogen (Table 1), e.g. a fish pathogen *Vibrio harveyi* can be detected by a specific primer set.

Multiplex Polymerase Chain Reaction (multiplex-PCR)

Multiplex polymerase chain reaction (Multiplex PCR) alludes to the utilize of polymerase chain response to intensify a few diverse DNA arrangements at the same time (as in case performing numerous isolated PCR responses all together in one reaction) (Kechin *et al.*, 2020; Markoulatos *et al.*, 2002). Multiplex-PCR was first described in 1988 as a method to detect deletions in the dystrophin gene (Chamberlain *et al.*, 1988). In multiplex PCR, two or more primer sets planned for the expansion of diverse targets are included within the same PCR reaction. (Shen, 2019a). This technique intensifies DNA in tests utilizing numerous primers and a temperature-mediated DNA polymerase in a warm cycler (Lorenz, 2012). The preliminary plan for all primer sets should be optimized so that all primer sets can work at the same annealing (strengthening) temperature amid PCR (Sint *et al.*, 2012). The multiplex polymerase chain reaction is a widespread molecular technique utilized for the

amplification of multiple targets in just one PCR experiment (Rollinson & Hay, 2012; Zebardast *et al.*, 2014).

Advantages of Multiplex- Polymerase Chain Reaction (multiplex-PCR)

In multiplex PCR Less input material is required and provides more information (Elnifro *et al.*, 2000). It is Cost effective (fewer dNTPs, enzymes, and other consumables) (Mahony *et al.*, 2009) and time-saving (versus conventional culture methods) (Giantsis *et al.*, 2017). It has increased accuracy of data analysis and fewer pipetting errors (Lee *et al.*, 2007). It can be used to identify exonic and intronic sequences in specific genes (Hernandez-Rodriguez, 2012).

Disadvantages of Multiplex- Polymerase Chain Reaction (multiplex-PCR)

The self-inhibition among different sets of primers can be occurred. In this PCR, it is important the design of primers because they must be characterized by adherence to specific DNA sequences at similar temperatures. However, it may require several trials to achieve the standardization of the procedure (Jackson *et al.*, 2004).

Application Multiplex- polymerase chain reaction (multiplex-PCR) in fish pathogen detection:

Numerous Multiplex- polymerase chain reaction (multiplex-PCR) assays are already examined and studied to detect the fish pathogenic bacteria and viruses (Table 2), such as *Aeromonas hydrophila*, *Edwardsiella tarda* and *Photobacterium damsela* can be detected in a single run of PCR by using multiple sets of primers. Fish pathogenic virus like infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV), and viral hemorrhagic septicemia virus (VHSV) can be identified by multiplex PCR (Williams *et al.*, 1999)

Real-Time Polymerase Chain Reaction (RT- PCR)

In the area of molecular diagnostics, real-time PCR-based assays have acquired favor in the recent past (Gunson *et al.*, 2006; Mackay, 2007). A real-time polymerase chain reaction (real-time PCR), additionally recognized as quantitative Polymerase Chain Reaction (qPCR), is a laboratory technique of molecular biology-based totally on the polymerase chain response (PCR) (Feroni *et al.*, 2017; García-Giménez *et al.*, 2019; Mauger & Deleuze, 2019). It observes the amplification of a targeted DNA molecule in the course of the PCR (i.e., in real-time), not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (quantitative real-time PCR) (Kralik & Ricchi, 2017). Real-time PCR is carried out in a thermal cycler with the ability to illuminate every sample with a beam of light of at least one particular wavelength and realize the fluorescence

Table 1. A shortlist of pathogen-specific primers used in polymerase chain reaction (PCR) methods to identify fish pathogens

Fish Pathogen	Host	Primer's name and sequence	References
<i>Vibrio parahaemolyticus</i>	Shrimp	Forward: AP4-F1- 5'ATGAGTAACAATATAAAAACATGAAAC3' Reverse: AP4-R1- 5'ACGATTTTCGACGTTCCCAA3' Forward: AP4-F2-5'TTGAGAATACGGGACGTGGG3' Reverse: AP4-R2 5'GTTAGTCATGTGAGCACCTTC3'	(Dangtip <i>et al.</i> , 2015)
Koi herpesvirus (KHV)	Koi, Goldfish, Common Carp	Forward: KHV-F-5'GACGACCCGGAGACCTTGTG3', Reverse: KHV-R- 5' CACAAGTTCAGTCTGTTCTCAAC3' Forward: KHV-TKf 5'GGGTTACCTGTACGAG3' Reverse: KHV-TKr 5' CACCCAGTAGATTATGC3' Forward: KHV-Gray-2F- 5'GACACCATCTGCAAGGAG3' Reverse: KHV-Gray-2R-5'GACACATGTTACAATGGTGGC3'	(Meyer <i>et al.</i> , 2012)
<i>Vibrio harveyi</i>	Sea bream	Forward: 5'GAGTTCGGTTCTTTCAAG3' Reverse: 5'TGTAGTTTTTCGCTAATTTTC3'	(Haldar <i>et al.</i> , 2010)
<i>Pseudomonas aeruginosa</i>	Freshwater culture system	Forward: 5'ATGGAAATGCTGAAATTCGGC3' Reverse: 5'CTTCTTCAGCTCGACGCGACG3'	(Tripathy <i>et al.</i> , 2007)
<i>Renibacterium salmoninarum</i>	Salmon	Forward: 5'GATCGTGAAATACATCAAG3' Reverse: 5'GGATCGTGTTT!ATCCACCC3'	(León <i>et al.</i> , 1994)
<i>Tenacibaculum soleae</i>	Senegalese sole fish, Wedge sole fish, Brill, Turbot	Forward: G47F- 5'ATGCTAATATGTGGCATCAC3' Reverse: G47R- 5'CGTAATTCGTAATTAACCTTTGT3'	(López <i>et al.</i> , 2011)
<i>Flavobacterium psychrophilum</i>	Rainbow trout, coho salmon, eel (<i>Anguilla anguilla</i>), Cyprinids, Pale chub	1 st step Forward: 20F- 5' AGAGTTTGATCATGGCTCAG3' Reverse: 1500R- 5' GGTTACCTGTTACGACTT3' 2 nd step Forward: PSY1-5'GTTGGCATCAACACACT3' Reverse: PSY2-5'CGATCCTACTTGCCTAG3'	(Wiklund <i>et al.</i> , 2000)
<i>Aeromonas salmonicida</i>	Salmonids	Forward: 27F-5'GAGTTTGATCTCGGCTCAG-3' Reverse: 1492r-5'TACGGYTACCTTGTACGACTT3' Forward: PAAS1 -5'CGTTGGATATGGCTCTTCCT -3' Reverse: PAAS2 -5'CTCAAACGGGTCGCTACCA3' Forward: AP1 -5'GGCTGATCTCTTCATCCTCACCC3' Reverse: AP2 -5' CAGAGTGAAATCTACCAGCGGTGC3' Forward: MIY1-5'AGCCTCCACGCGCTCACAGC3' Reverse: MIY2-5'AAGAGCCCCATAGTGTGGG3'	(Byers <i>et al.</i> , 2002)
<i>Tenacibaculum soleae</i>	Turbot, Dover sole, Senegalese sole, Sea bass, Atlantic salmon, Coho salmon	Forward: Sol-Fw-5'TGCTAATATGTGGCATCAAA3' Reverse: Sol-Rv-5' CAACCCATAGGGCAGTCATC3'	(García-González <i>et al.</i> , 2011)
<i>Vibrio anguillarum</i>	Several fishes	Forward: van-ami8-5' ACAT CATCCATTTGTTAC3' Reverse: van-ami417-5' CCTTATCACTATCCAAATTG3'	(Hong <i>et al.</i> , 2007)
<i>Flavobacterium psychrophilum</i>	Rainbow Trout, Salmonids	Forward: FP1 -5' GTTAGTrGGCATCAACAC3' Reverse: FP2-5'TCGATCCTACTTGCCTAG3' Forward: 5'AGAGTTTGATCATGGCTCAG3' Reverse: FP2-5'CGGTACCTTGTACGACTT3'	(Urdaci <i>et al.</i> , 1998)
<i>Myxobolus cerebralis</i>	Salmonids, Brown Trout, Rainbow Trout, Gibel Carp	Forward: Tr5-17-5'GCCTATTAAGTGTGGTAGTATAGAAGC3' Reverse: Myx18-996R-5'GCGGTCTGGGCAAATGC3' Forward: Tr3-17-5'GGCACACTACTCCAACACTGAATTTG3' Reverse: Myx18-909f-R-5'TGCTGTAAGTGAATAACATTCAGTCAAAG3'	(Andree <i>et al.</i> , 1998; Eszterbauer <i>et al.</i> , 2019; Kelley <i>et al.</i> , 2004)
<i>Sphaerothecum destruens</i>	Salmonids	Forward: Sd-1F-5'CGACTTTTCGGAAGGGATGATT3' Reverse: Sd-1R -5'AGTCCCAAACCTCGACGACACT3' Forward: Sd-2F -5'CCCTCGGTTTCTGGTGATTCAATAACT3' Reverse: Sd-2R -5'CTCGTCGGGGCAAACACCTC3'	(Mendonca & Arkush, 2004)
<i>Amyloodinium ocellatum</i>	Several marine fishes	Forward: Dino5'UF-5'CAACCTGGTGATCTGCCAGT3' Reverse: ITSr -5'TCCCTGTTTCATTCGCCATTAC3' Forward: AO18SF1 -5'GACCTTGCCGAGAGGG3' Reverse: AO18SR1-5'GAAAGTGTGGTGAATCTTAAC3'	(Levy <i>et al.</i> , 2007)
<i>Myxobolus pseudodispar</i>	Cyprinid fish	Forward: Tub16SF-5'AACGGCCGCGGTATCCTG3' Reverse: Tub16SR-5'TAARCCAACATYGAGGTGCCA3' Forward: MpF1-5'TGTGCTTCTGGTGCCTGCTGC3' Reverse: PseudoR-5'AAGCACCGAAGCACAGTCAA3'	(Beauchamp <i>et al.</i> , 2001; Marton & Eszterbauer, 2012)
Ascaridoidea (<i>Anisakis pegreffii</i> , <i>A. physeteris</i> , <i>A. simplex</i> , <i>Contraecaecum osculatum</i> , <i>C. radiatum</i> , <i>C. rudolphi</i> , <i>Hysterothylacium aduncum</i> <i>Porrocaecum angusticolle</i> , <i>P. crassum</i> , <i>P. depressum</i> , and <i>P. ensicaudatum</i>)	Several freshwater and marine fishes	Forward: NC5-5'GTAGGTGAACCTGCGGAAGGATCATT3' Reverse: NC2-5'TTAGTTTCTTTCTCCCTCGCT3'	(Kijewska <i>et al.</i> , 2002; Zhu <i>et al.</i> , 1998)
Anisakid Nematodes	Black scabbardfish, Chub mackerel, Blue jack mackerel	Forward: Primer A- 5'GTCGAATTCGTAGTGAACTGCGGAAGGATCA3' Reverse: Primer B1-5'GCCGGATCCGAATCCTGGTTAGTTTCTTTCTC3'	(Pontes <i>et al.</i> , 2005)
<i>Gyrodactylus</i> sp.	Rainbow trout, Brown trout, Salmon, Grayling	Forward: ITS1F-5'GTTTC CGTAG GTGAA CCT3' Reverse: ITS2R-5'GGTAA TCACG CTTGAATC3'	(Rokicka <i>et al.</i> , 2007)

emitted by using the excited fluorophore (Ahrberg *et al.*, 2016). The PCR technique usually consists of a sequence of temperature changes that are repeated 25-50 times (Chen *et al.*, 2019). The recommended temperature and the duration for RT-PCR used for every cycle depend on a variety of parameters and factors, such as the concentration of divalent ions, the enzyme used to synthesize the DNA, deoxyribonucleotides (dNTPs) in the reaction, and the annealing temperature of the primers, etc. (Sambrook, 2001; Shaheen Shahzad *et al.*, 2020; van Pelt-Verkuil *et al.*, 2008).

Advantages of Real-Time Polymerase Chain Reaction (RT- PCR)

Real-time PCR is not influenced by non-specific amplification (Dorak, 2007). This assay required less amount of the template material (Stahlberg *et al.*, 2005). The major advantage over the other PCR technique is the quantification and it quantifies the template DNA or RNA present in the sample (Tom *et al.*, 2004). By this method, amplification can be monitored in real-time (Mackay *et al.*, 2002) and it enables high

Table 2. A shortlist of Multiplex Polymerase chain reaction (multiplex-PCR) assay used to detect fish pathogens.

Host	Fish pathogen	Target gene of pathogen	Primer name and sequences	References
Sea bream	Red sea bream iridoviruses (RSIV)	<i>RNRS gene</i>	Forward: VF- 5'GCATGTATGCTGTTTAGACA3' Reverse : VR- 5'GAGCATCAAGCAGGCGATCT3'	(Jeong <i>et al.</i> , 2004)
		<i>ATPase gene</i>	Forward: 3F- 5'CAAACCACAGCGCGCAAGT3' Reverse : 3R- 5'AGTAGCGCACCATGTCCTCC3'	
		<i>DPOL gene</i>	Forward: 4F- 5'CGGGGCAATGACGACTACA3' Reverse : 4R- 5'CCGCCTGTGCCTTTTCTGGA3'	
		<i>Pst I fragment</i>	Forward: 1F- 5'CTCAAACACTCTGGCTCATC3' Reverse : 1R- 5'GCCAACACATCTCCTATC3'	
Rainbow trout	<i>Lactococcus garvieae</i>	<i>Internal transcribed spacer gene (ITS)</i>	Forward: Lg F- 5'ACTTTATTAGTTTTGAGGGTCT3' Reverse : Lg R- 5'TTAACTGCTTCGTTGACCAGA3'	(Chapela <i>et al.</i> , 2018; Keeling <i>et al.</i> , 2012; Marancik & Wiens, 2013)
	<i>Yersinia ruckeri</i>	<i>glnA gene</i>	Forward: YR glnA F-5'TCCAGCACCAATACGAAGG3' Reverse : YR glnA R-5'ACATGGCAGAACGCAGATC3'	
	<i>Flavobacterium psychrophilum</i>	<i>Unknown protein</i>	Forward: Fp Sig F-5'GGTAGCGGAACCGAAATG3' Reverse : Fp Sig R-5'TTTCTGCCACCTAGCGAATACC3'	
Yellow croaker	<i>Vibrio alginolyticus</i>	<i>Collagenase gene</i>	Forward: VA-F-5'CGAGTACAGTCACTTGAAGCC3' Reverse : VA-R-5'CACAACAGAAGCTCGGTTACC3'	(Liu <i>et al.</i> , 2016)
	<i>Vibrio parahaemolyticus</i>	<i>Collagenase gene</i>	Forward: VP-F-5'GAAAGTTGAACATCAGCACGA3' Reverse : VP-R-5'GGTCAGAATCAAACGCCG3'	
	<i>Vibrio harveyi</i>	<i>ToxR</i>	Forward: VH-F-5'GAAGCACTCACCGAT3' Reverse: VH-R-5'GGTGAAGACTCAGCA3'	
Tilapia , Japanese eel, Flounders	<i>Aeromonas hydrophila</i>	<i>16s rRNA</i>	Forward: Ah-F- 5'GTAATGCCTGGGAAAT3' Reverse : Ah-R- 5'TTGATACGTATAGGCA3'	(Chang <i>et al.</i> , 2009)
	<i>Edwardsiella tarda</i>		Forward: Et-F- 5'ACGTAACGTCGCAAGA3' Reverse : Et-R- 5'CCGTGTATATACAGGA3'	
	<i>Photobacterium damsela</i>		Forward: Pd-F- 5'GCTTGAATACATTGAG3' Reverse : Pd-R- 5'CACCTTTCGGTCTTGCT3'	
	<i>Streptococcus iniae</i>		Forward: Si-F- 5'CACTAATCCAAAGAGTT3' Reverse : Si-R- 5'TTAGCGGCTGGCTCCTAA3'	
Rainbow trout	<i>Renibacterium salmoninarum</i>	unknown	Forward: Rs1-5'CAAGTGGAAGGGAATTCTTCCACT3' Reverse: Rs2-5'GACGGCAATGTCCGTTCCCGGTT3'	(Altinok <i>et al.</i> , 2008; Brown <i>et al.</i> , 1994)
	<i>Aeromonas hydrophila</i>		Forward: AH1-5'GAAAGGTTGATGCCTAATACGTA3' Reverse: AH2-5'CGTGCTGGCAACAAAGGACAG3'	
	<i>Yersinia ruckeri</i>		Forward: YerF-5'GCGAGGAGGAAG GGTAAAGTG3' Reverse: YerR-5'GAA GGCACCAAGGCATCTCTG-3'	
	<i>Aeromonas salmonicida</i>		Forward: ASF-5'CGTTGGATATGGCTCTTCT3' Reverse: ASR-5'CTCAAACGGCTGCGTA3'	
	<i>Flavobacterium columnare</i>		Forward: FCF-5'AAGGCAACGATGGGTAG3' Reverse: 161B-5'GCACGGAGTTAGCCGATC3'	
Brown trout, grayling, pike, and whitefish	Infectious Pancreatic Necrosis Virus (IPNV)	<i>VP2 gene</i>	Forward: WB1-5'CCGCAACTACTTGAGATCCATTATGC3' Reverse: WB2-5'CGTCTGGTTCAGATTCCACCTGTAGTG3'	(Williams <i>et al.</i> , 1999)
	Infectious Hematopoietic Necrosis Virus (IHNV)	<i>N gene</i>	Forward: IHN3-5GTTCAACTTCAACGCCAACAGG3' Reverse: IHN4-5'TGAAGTACCCACCCGAGCATCC3'	
	Viral Hemorrhagic Septicemia Virus (VHSV)	<i>G gene</i>	Forward: VHS3-5'CGGCCAGCTCAACTCAGGTGTCC3' Reverse: VHS4-5'CCAGGTCGGTCTGATCCATTCTGTC3'	

confidence detection of low copy targets. It needs no post PCR processing (Hanna *et al.*, 2005).

Disadvantages of Real-Time Polymerase Chain Reaction (RT-PCR)

Real-time PCR (RT-PCR) technique will only indicate the presence of antigenic material during infection and will not indicate if a host was infected. It needs specialized bio-containment laboratories, operated by highly trained technicians, which makes it an expensive test and difficult to scale. Real-time PCR (RT-PCR) kits are not available for all kind of genes and disorders and the technical and standardized protocols are limited. Furthermore, higher expertise and technical skills are required for developing an RT-PCR assay.

Application Real-Time Polymerase Chain Reaction (RT-PCR) in Fish Pathogen Detection

RT-PCR has been increasingly utilized to identify fish pathogens (both virus and bacteria) since the beginning of the twenty-first century (Table 3), e.g. Tilapia lake virus (TiLV) can be detected by real-time PCR.

Random Amplified Polymorphic DNA (RAPD)

It is a PCR-based technique, however, the difference is segments of DNA that are randomly amplified (Kumari & Thakur, 2014; Zia *et al.*, 2020). The scientist performing RAPD creates numerous arbitrary, short primers normally 10 bp (range can be 8-12 nucleotides sometimes more than that) (Rocco *et al.*, 2014; Shekhawat *et al.*, 2019), then operated with the PCR, the usage of a large template of genomic DNA, hoping that fragments will be amplified (Arora *et al.*, 2013). By resolving the resulting patterns, a semi-unique to a unique profile can be picked up from an RAPD reaction (Galanis *et al.*, 2015). Unlike standard PCR analysis, RAPD does no longer requires any precise understanding of the DNA sequence of the target organism (Galanis *et al.*, 2015; Martín *et al.*, 2014). The segment of DNA, whether it will be amplified or not, it depends on positions of primers (generally identical 10-mer) which are complementary to DNA sequences (Premkrishnan & Arunachalam, 2012). For example, no fragment will produce if primers are annealed too far distance from opposite annealed primer or they are not facing each other which means they are in the same direction (Clark *et al.*, 2019; Hommelsheim *et al.*, 2014).

Advantages of Random Amplified Polymorphic DNA (RAPD)

It requires no DNA probes and sequence information for the design of specific primers (Hadrys *et al.*, 1992). It involves no blotting or hybridization steps, hence, it is quick, simple and efficient (Kumar &

Gurusubramanian, 2011). It requires only small amounts of DNA (about 10 ng per reaction) and the procedure can be automated (Kumar & Gurusubramanian, 2011). It produces a high number of fragments (Rieseberg, 1996). Additional findings supported the use of RAPD analysis as an effective tool in species identification and cross-contamination test among different cell lines (Guo *et al.*, 2001). The RAPD-PCR method can be applied to detect genetic diversity and similarity in numerous organisms using various primers (Andrighetto *et al.*, 2001; Berthier & Ehrlich, 1999). For all of these reasons, the RAPD assay has been used to construct phylogenetic trees for resolving taxonomic problems in many organisms (Chalmers *et al.*, 1992).

Disadvantages of Random Amplified Polymorphic DNA (RAPD)

Nearly all RAPD markers are dominant (Lynch & Milligan, 1994), i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Codominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely (Kumar & Gurusubramanian, 2011). The RAPD technique is notoriously laboratory-dependent and needs carefully developed laboratory protocols to be reproducible (Caliskan, 2012). Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product (Caliskan, 2012). RAPD results can be difficult to interpret and lack of prior knowledge on the identity of the amplification products. It has problems with reproducibility (sensitive to changes in the quality of DNA, PCR components and PCR conditions). Gel electrophoresis can separate DNA quantitatively, cannot separate equal-sized fragments qualitatively (i.e. according to base sequence) (Nandani & Thakur, 2014).

Application RAPD in Fish Pathogen Detection

Comparing to other PCR-based molecular diagnostic methods, a few RAPD has been studied to identify fish pathogens (Table 4), e.g. marine fish pathogen *Tenacibaculum maritimum* can be identified by using RAPD.

Nested PCR

Nested PCR is an improved adaptation of PCR designed to enhance the sensitivity and specificity of the procedure (Shen *et al.*, 2019; Carr *et al.*, 2010). At first Kamolvarin (1993) described the nested PCR method. Nested PCR entails the employment of two primer sets and two PCR reactions in succession. The first set of primers is used in an initial PCR reaction and is designed to anneal to sequences upstream from the second set of primers. The first PCR reaction's amplicons are utilized

Table 3. A shortlist of real time polymerase chain reaction (RT-PCR) assay used for fish pathogen identification

Fish pathogen	Host	Primer name and sequences	References
Infectious myonecrosis virus (IMNV)	Shrimp	Forward: IM-MCP3571-3592F- 5'CGCGCCAGCTTTCCATTAGT3' Reverse: IM-MCP3688-3706R- 5'CCAATGTTTCGAGCACCTC3' Forward: IM-MCP3572-3593F- 5'GCGCCAGCTTTCCATTCAGTG3' Reverse: IM-MCP3698-3716R- 5'CCAATGTTTCGAGCACCTC3'	(Kokkattunivarthil et al., 2018)
Tilapia lake virus (TiLV)	Tilapia, White leg shrimp	Forward: Nested ext-1- TATGCACTACTTTCCCTGCC3' Forward: ME1- 5' GTTGGGCAACAAGGCATCTA3' Reverse: 7450/150R/ME2- 5'TATCACGTGCGTACTCGTTCAGT3' Forward: TiLV-Seg1-F- 5'TCATTCCGCTATATAGTTAC3' Reverse: TiLV-Seg1-R- 5'TTAATTACGCACTATTACTG3' Forward: TiLV-Seg5-F- 5'TTTTCTCAGTTTACCACTC3' Reverse: TiLV-Seg5-R- 5'TTATCTCAGACTCCAATAGC3' Forward: TiLV-Seg9-F- 5'AGTCCTTAAAGTCATACTT3' Reverse: TiLV-Seg9-R- 5'ACAAGTCCGATTACTTTTTC3'	(Dong et al., 2017; Eynor et al., 2014, 2014)
Viral hemorrhagic septicemia virus (VHSV)	Olive flounder, Rainbow trout, Turbot	Forward: VHSV-Universal-F- 5'GWGGAGARGGACGGTCTGT3' Reverse: VHSV- universal-R- 5'TCTGTCACCTTGATCCCTCCAG3' Forward: VHSV-I-F- 5'AGAGTGACTTATCGAGTCACCTGTCCG3' Reverse: VHSV-I-R- 5'GAACAGGTGCTTCTAGTGTTCGAC3' Forward: VHSV-IV-F- 5'TCCATCCTTATTTCCATGAGAGGAGAAGGA3' Reverse: VHSV-IV-R- 5'TCCAGTAGACTCCTTGCCAGTGGGTTG3'	(Hwang et al., 2018)
<i>Vibrio harveyi</i>	Seabass, shrimps, mollusks, crustaceans	Forward: mreB11F- 5'TGAAGCTGTGATCAACTACG3' Reverse: mreB9bisR- 5'CCTGACAGTGGCTCTTGTA3' Forward: Vh. topA-F- 5'TGGCGCAGCGTCTATACG3' Reverse: Vh.topA-R- 5'TATTTGTCACCGAAGTCAAGACC3' Forward: toxRF1- 5'GAAGCAGCACTCACCGAT3' Reverse: toxRR1- 5'GGTGAAGACTCATCAGCA3'	(BAILLIE et al., 2015; Mouglin et al., 2020; Pang et al., 2006)
<i>Streptococcus agalactiae</i>	Nile tilapia	Forward: 5' CATTGCGTCTTGTAGTTTGTAG3' Reverse: 5' GGAGCCTAGCGGATCGA3'	(Asencios et al., 2016)
<i>Enteromyxum scophthalmi</i>	Turbot	Forward: ENTEROMYX F- 5'GGCTTAATTTGACTCAACA3' Reverse: ENTEROMYX R- 5'TCCACCAACTAAGAACG3'	(Alonso et al., 2015)
<i>Francisella noatunensis subsp. orientalis</i>	Tilapia, Atlantic cod Atlantic Salmon, Striped bass, Ornamental cichlids	Forward: iglC- 5'GGCGTATCTAAGGATGGTATGAG3' Reverse: iglC-5'AGCACAGCATACAGGCAAGCTA3' Probe: FAM-5'ATCTATTGATGGGCTCACAACTTCAAAA' BHQ-1	(Soto et al., 2010)
<i>Ichthyophthirius multifiliis</i>	Several freshwater fishes	Forward: IMRf1- 5' AGTGACAAGAAATAGCAAGCCAGGAG3' Reverse: IMRr1-5' ACCCAGCTAAATAGGCAGAAAGTTCAA3'	(Jousson et al., 2005)
<i>Ceratomyxa shasta</i>	Salmonid fishes	Forward: Cs-1034F- 5'CCAGCTTGAGATTAGCTCGGTAA3' Reverse: Cs-1104R-5'CCCCGGAACCCGAAAG3' Probe: 1058T 6-FAM-CGAGCCAAGTTGGTCTCT CCGTAAAACTA-MRA	(Hallett & Bartholomew, 2006)
Anisakids (nematodes)	Several marine fishes	Forward: COI F- 5'GGKCYATTAAYTYTATRACWACTAC3' Reverse: COI R -5'AAAGAWGTATTMARRTTACGRTCVG3' Probe: COI PROBE- FAM-TCTATTCTTTGGARCAAYA-TAM	(Herrero et al., 2011)
<i>Ichthyobodo</i> spp.	Salmon, Halibut, and several fishes	Forward: CosF1-5'AATAGGAGGTCTGCGAACG3' Forward: CosF2-5'CCTGAGAAACAGTACCCT3' Reverse: CosR4-5'CCGAGCGGTCTAAGATTTC3' Reverse: CosR5-5'TTCCTGTAAGTAAAGTTCC3' Forward: Costia F-5'ACGAACTTATGCGAAGGCA3' Reverse: Costia R-5'TGAGTATTCACTYCCGATCCAT3' Probe: Costia (FAM)- 5' TCCACGACTGCAAACGATGACG-3' (TAMRA)	(Isaksen et al., 2012)
<i>Myxobolus cerebralis</i>	Rainbow trout, Salmonid	Forward: Tr5-3-5'CGTGAGACTGCGGACGGCTCAG3' Reverse: Tr3-1-5'CGGTGTGTACAAAAGGGCAGGGAC3' Forward: Tr5-6-5'GGCAGCGTTAAACTGTCTCACG3' Reverse: Tr3-6-5'CCTCACAGTCTCTCATGACAC3' Forward: MX5-5'CTGCGGACGGCTCAGTAAATCAGT3' Reverse: MX3-5'CCAGGACATCTTAGGGATCAGACA3' Forward: Tr5-16-5'GCATTGGTITACGCTGATGTAGCGA3' Reverse: Tr5-17-5'GCCCTATAACTAGTTGGTAGTATAGAAGC3' Forward: Tr3-16-5'GAATCGCCGAAACAATCATCGAGCTA3' Reverse: Tr3-17-5'GGCACACTCCAACACTGAMTTG3'	(Andree et al., 1997, 1998; Kelley et al., 2006)
<i>Thelohanellus kitauei</i>	Carp	Forward: UEP-F-5'ACCTGGTTGATCCTGCCAG3' Reverse: UEP-R-5'CTTCCGAGGTTACCTACGG3' Forward: TkF-1-5'GCCAGTAATCTACTATTCCGACG3' Reverse: TkR-1-5'GCTATTGATCTGTTAATCCTATC3'	(Barta et al., 1997; Seo et al., 2012)
<i>Anisakis pegreffii</i>	Crustaceans (Krill), Fish and Squids	Forward: RTpegF-5'CTTTGGAGGTTGATAATCG3' Reverse: RTpegR-5'CCCAAAATCTCTGAACATT3' Probe: pegHyPr-5'CTTGGGCTTTGGCTAGGATGTG3' (6FAM/BHQ)	(Paoletti et al., 2018)
<i>A. simplex</i>		Forward: RTsimF-5'CTTTAATTTGGTTGCTCAGAT3' Reverse: RTsimR-5'CGATTATCAACCTCCAAAAG3' Probe: simHyPr-5'ATGACCAGTGACTTTCACAGTCAAAT3' (5-CY/BBQ)	
<i>Pseudoterranova decipiens</i>		Forward: RTdecF-5'GGCTTGATAAAATTTGGACAG3' Reverse: RTdecR-5'ATAAAATACCTCTCATAGCATCC3' Probe: decHyPr-5'CTTCCGGCGATGTAATTCAT3' (YAK/BHQ)	
<i>P. bulbosa</i>		Forward: RTbulF-5'CTGGTCTCATGTTGATTGA3' Reverse: RTbulR-5'CCTCTCATAGCATCCAACCT3' Probe: bulHyPr-5'ACAACCGTTGTGTTCTCT3' (6FAM/BHQ)	
<i>P. krabbei</i>		Forward: RTkraF-5'GTTGATCAGTTGGAGTTGG3' Reverse: RTkraR-5'CAAATCTCAGAACACTGACC3' Probe: kraHyPr-5'TTCTGGGCTTGCCTAGAA3' (LC640/BHQ)	
<i>Hysterothylacium aduncum</i>		Forward: RThystF-5'ATTTGACTATCAAGGTAAGTGGT3' Reverse: RThystR-5'TTAATAGCCATTCTAGGCAAAA3' Probe: hystHyPr-5'GATCACACAACGGTTATCCACTCT3' (YAK/BHQ)	

as a template for a second set of primers and a second amplification step (Shen *et al.*, 2019). With this approach, the sensitivity and specificity of DNA amplification might be greatly improved.

Advantages of Nested PCR

The first and most efficient point of nested PCR is that the specificity and sensitivity were higher than those of conventional PCR methods (Liop *et al.*, 2000). Another benefit of nested PCR is that it uses less target DNA (Liop *et al.*, 2000). It can also help with the amplification of low-abundance genes.

Disadvantages of Nested PCR

However, adding a second amplification step and manipulating previously amplified material at the same time could result in a large rise in false positives due to cross contamination, making this strategy too precarious for routine analysis (Liop *et al.*, 2000).

Application of Nested PCR in Fish Pathogen Detection

Nested PCR has been used as a viable tool for the detection of pathogens causing fish diseases where a single round of PCR is insufficient. Researchers around the globe have successfully developed nested PCR protocols for the identification of perilous fish pathogens including *Vibrio vulnificus*, *Ranibacterium salmoninarum*, *Flavobacterium psychrophilum*,

Flavobacterium columnare, *Tenacibaculum maritimum*, *Photobacterium damsela*, *Myxobolus cerebralis* (Andree *et al.*, 1998), *Tenacibaculum maritimum* (Avenidaño-Herrera *et al.*, 2004), *Flavobacterium columnare* (Bader *et al.*, 2003), *Enterocytozoon salmonis* (Barlough *et al.*, 1995), *microsporidium seriolae* (Bell *et al.*, 1999), *Flexibacter maritimus* (Cepeda *et al.* 2003), *Cyprinid herpesvirus-3* (El-Matbouli *et al.*, 2007), *Flavobacterium psychrophilum* (Izumi *et al.*, 2005), *Pseudomonas plecoglossicida* (Izumi *et al.*, 2007), *Sphaerothecum destruens* (Men- donca and Arkush 2004), *Myxobolus cerebralis* (Skirpstunas *et al.*, 2006), *Flavobacterium psychrophilum* (Wiklund *et al.*, 2000) etc (Jimenez *et al.*, 2011; Baliarda *et al.*, 2002; Osorio *et al.*, 1999; Osorio *et al.*, 2000; Chase and Pascho, 1998; Arias *et al.*, 1995)

Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a DNA amplification technique introduced in the year 2000 that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions (Notomi *et al.*, 2000). The LAMP reaction uses a DNA polymerase with high strand displacement activity and pairs of specially designed inner and outer primers. At the three prime terminal, each of the inner primers has a sequence that is complementary to one chain of the amplification region and identical to the inner region of the same chain at the five prime terminal. Using the aforementioned stem loop areas as a stage, DNA

Table 4. A short list of Random Amplified Polymorphic DNA (RAPD) assay used for fish pathogen identification

Host	Pathogen	Primer name and sequences	References
Sea bream, Sea bass	<i>Photobacterium damsela</i> subsp. <i>piscicida</i> (<i>Vibrionaceae</i>)	RAPD: AA- 5'GGTGTCGGGCTACCACTACGGG3' RAPD: BC- 5'ACCACTTCGGGTTTCATGCC3'	(Dalla Valle <i>et al.</i> , 2002)
Salmonid fish	<i>Aeromonas salmonicida</i>	1 st step, RAPD: A05-5'AGCAGCGCCTCA3' 2 nd step, Forward primer: 5'AGCCTCCACGCGCTCACAGC3' Reverse primer: 5'AAGAGCCCCATAGTGTGGG3'	(Inglis & Aoki, 1996)
Yellowtail, Rainbow trout	<i>Lactococcus garvieae</i>	1 st step, Forward primer: PLG-1- 5'CATAACAATGAGAATCGC3' Reverse primer: PLG-2- 5'GCACCCTCGGGTTG3' 2 nd step, RAPD: P1-5'GGTGCGGGAA3', P2- 5'GTTTCGCTCC3', P3- 5'GTAGACCCGT3', P4- 5'AAGAGCCCGT3', P5- 5'AACGCGCAAC3', P6- 5'CCCGTCAGCA3'	(Ravelo <i>et al.</i> , 2003)
Turbot, Salmon, Sole, Gilthead seabream	<i>Tenacibaculum maritimum</i>	RAPD: P1- 5'GGTGCGGGAA3', P2- 5'GTTTCGCTCC3', P3- 5'GTAGACCCGT3', P4- 5'AAGAGCCCGT3', P5- 5'AACGCGCAAC3', P6- 5'CCCGTCAGCA3'	(Avenidaño-Herrera <i>et al.</i> , 2004)
Trout, Salmon	<i>Aeromonas salmonicida</i> and <i>Aeromonas hydrophila</i>	RAPD: A05- 5'AGCAGCGCCTCA3', A07- 5'TGCCTCGACCA3', A09- 5'CCGAGTTAGAT3'	(Miyata <i>et al.</i> , 1995)
Rainbow trout, Coho salmon	<i>Flavobacterium psychrophilum</i>	RAPD: Primer 1- 5'TTCGAGATCCCAACAACA3', Primer 2- 5'CTAAGTACCGCCCGATC3'	(Crump <i>et al.</i> , 2001)
Trout and Carp	<i>Argulus</i> sp.	RAPD: OPA4-5'AATCGGGCTG3', OPA11-5'CAATCGCCGT3', OPA14- 5'TCTGTGCTGG3', OPC11-5'AAAGCTGCGG3', OPC15- 5'GACGGATCAG3', OPC19-5'GTTGCCAGCC3', OPH11- 5'CTTCCGAGT3', OPY2-5'CATCGCCGCA3', OPY20- 5'AGCCGTGGAA3'	(Sahoo <i>et al.</i> , 2013)

polymerase-mediated strand-displacement synthesis repeats the elongation events in a sequential manner. This approach works on the basis of producing a large number of DNA amplification products with a mutually compatible sequence and an alternating, repeating structure.

Advantages of LAMP

LAMP is very easy and simple method to execute as soon as the suitable primers are prepared, requiring a DNA polymerase, four primers and a regular laboratory water bath or heat block for reaction. Moreover, it is extremely precise for the target sequence and amplifies DNA with high efficiency producing 10^9 copies of target sequence less than an hour. Another benefit of LAMP is that it may efficiently amplify RNA sequences when used in conjunction with reverse transcription.

Disadvantages of LAMP

LAMP is less versatile than conventional PCR. Kermekchiev et al. (2009) found lower sensitivity of LAMP than PCR in case of complex sample like blood. Due to difficulty in proper primer designing, LAMP is not useful for cloning purposes but for useful as a diagnostic or detection technique (Torres et al., 2011).

Application of LAMP in Fish Pathogen Detection

In the investigation of fish diseases, LAMP has been employed frequently due to its advantageous context over other PCR based techniques. Rapid screening of different fish pathogenic bacteria including *Edwardsiella ictaluri* (Yeh et al., 2005), *Flavobacterium columnare* (Yeh et al., 2006), *Nocardia seriolae* (Itano et al., 2006), *Vibrio anguillarum* (Kulkarni et al., 2009; Gao et al., 2010) *Yersinia ruckeri* (Saleh et al., 2008), *Lactococcus garvieae* (Tsai et al., 2013), *Streptococcus inae* (Zhou et al., 2018), *Myxobolus cerebralis* (El-Matbouli and Soliman 2005a), *Tetracapsuloides bryosalmonae* (El-Matbouli and Soliman 2005b), koi herpes virus (Gunimaladevi et al., 2004), hematopoietic necrosis virus (Gunimaladevi et al., 2005), *Edwardsiella tarda* (Savan et al., 2004), spring viraemia of carp virus (Shivappa et al. 2008), viral hemorrhagic septicaemia virus (Soliman et al., 2006) were executed engaging LAMP technique.

Conclusion

In case of disease outbreaks, quick diagnosis and rapid removal of infected fish are necessary for the successful implementation of effective control and health management strategies in aquaculture. Past diagnostic methods tend to be expensive, labor-intensive, time-consuming, and might not lead to a specific diagnosis, even when compared with

histological evidence. PCR-based molecular diagnostic methods help to prevent disease outbreaks and reducing the potentiality of producing antibiotics resistant microorganisms. It has the capacity for rapid, sensitive diagnosis even it can detect pathogen from asymptomatic fishes. The main aim of the PCR-based molecular diagnostic methods are epidemiological studies as well as identifying causes of disease outbreaks and identifying the presence of pathogens. In recent years, the number of new publications describing new molecular techniques has increased significantly. These publications demonstrate the development of new molecular diagnostic methods that appear very promising and useful. This is the high time to wide-scale adoption and application of PCR based molecular diagnostic methods.

Ethical Statement

This study does not require ethical statement.

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Author Contribution

MAAA has searched and contributed in writing this article. MAAA, MSH, SIP, JC, RE and AR have designed the paper structure and contributed to editing and revising the article. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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