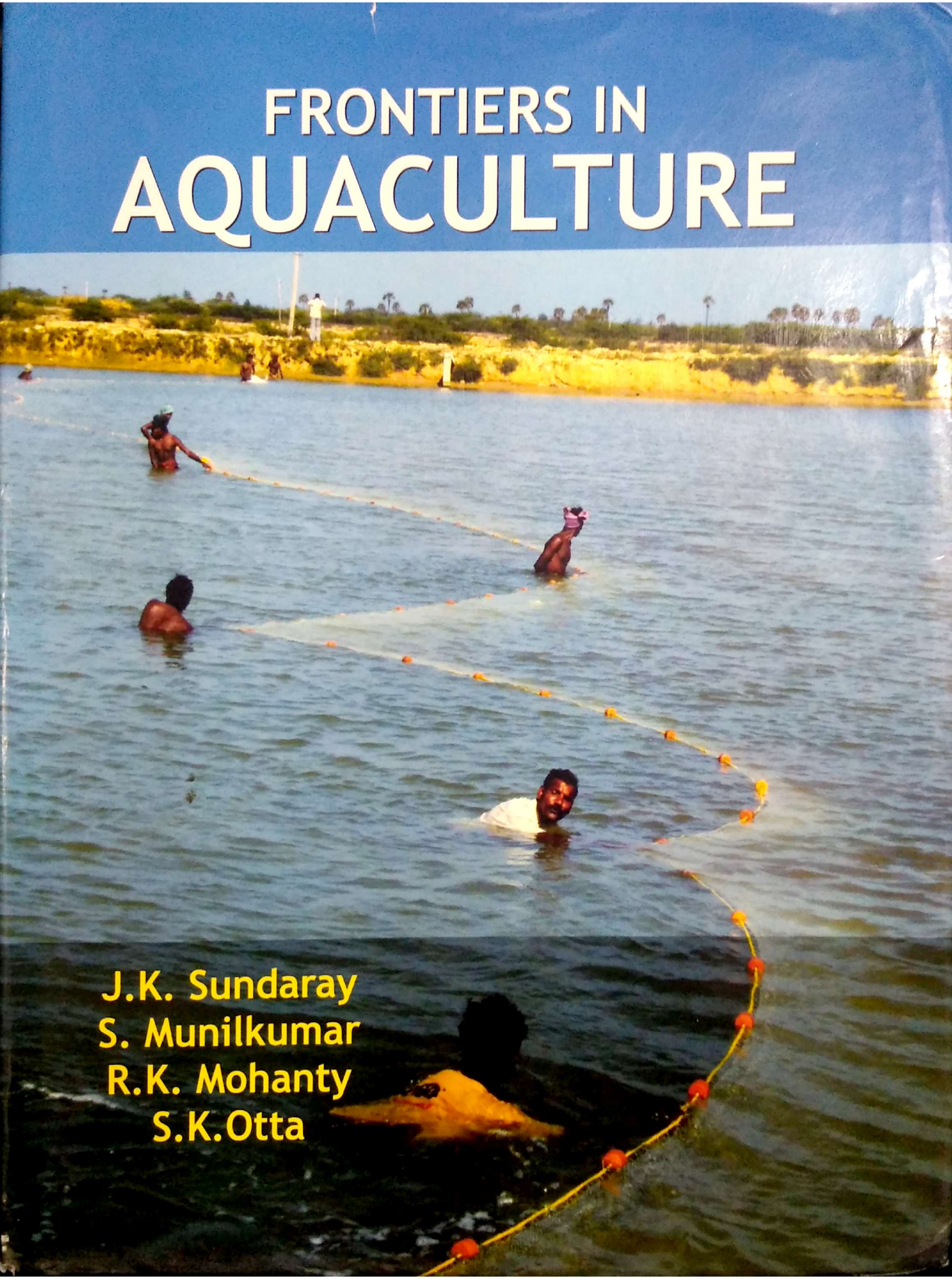
FRONTIERS IN AQUACULTURE



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ADVANCES IN MOLECULAR DIAGNOSTICS AND THERAPEUTICS IN AQUACULTURE RESEARCH

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INTRODUCTION

Nucleic acid-based techniques for the detection of causative agents and to avoid their multiplication in the host cells have gained immense importance because of their specificity and sensitivity. The pace at which publications appear by the use of these techniques for the identification of viral and bacterial pathogens of fish and shrimp seems to be ever-increasing. This is inspite the fact that, the adoption of these techniques has been much slower than had been expected. This chapter is aimed at not to provide an exhaustive review of all the methods available since it would quickly be dated and perhaps obsolete by the ongoing developments. Therefore,, it is focused to highlight some of the specific areas where molecular approach have been successful in addition to some of the lacunae that they have not yet been adopted as expected and some future developments in the molecular therapeutics.

Polymerase Chain Reaction (PCR)

One of the very fine discoveries in science is the structure of nucleic acids (Watson and Crick, 1953) which led to the further development in the field of molecular biology. It has been the most significant achievement for the molecular diagnosis after the advent of PCR (Mullis and Faloona, 1987; Saiki et al., 1998). The *in vitro* amplification of DNA and generation of complementary DNA (cDNA) by reverse transcription of RNA was already tried several years prior to the invention of PCR (Arya and Young, 1980). Mostly, short stretches of nucleic acid that are unique to the target organisms are amplified and the very presence of it is considered as a sufficient evidence for the presence of the target pathogen. Frequently, fish pathogens are only the distant relatives of those organisms that have already been studied in detail. Therefore, primers designed to detect fish pathogens by PCR have every possibility to cross-react with the closely related organisms

and give false positive results. As the genome of every single organism has not yet been sequenced, specificity cannot be fully guaranteed, though the chances of false positives can be avoided to a great extent by the stringent primer design procedures targeting the regions of the genome that are likely to be specific only to the organism taken for the detection. Efforts are still on to find out a fast and sensitive PCR methods for the detection of pathogen of aquatic organisms (Mortem *et al.*, 2010). The use of nested PCR protocols can improve the sensitivity of detection. When this is not possible, less specific amplification reactions can still be extremely valuable, particularly when dealing with a novel or poorly studied pathogen. In these cases, post-amplification analyses improve the specificity of the test.

Other PCR related techniques

The product can be further analyzed combined with other amplification methods like nucleic acid sequence-based amplification (NASBA) which has advantage over conventional PCR (Birch *et al.*, 2001; Niesters, 2002) but has not yet been widely applied for the diagnosis of aquaculture related diseases. Random amplified polymorphic DNA (RAPD) is a modification of PCR that can potentially scan the whole genome to reveal variation, rather than targeting a small portion for examination (Williams *et al.*, 1990; Welsh and McClelland, 1990). While developing diagnostic tests for fish pathogens, RAPD is probably best used as a first step to identify polymorphic DNA, from which primers or probes can be designed for a more robust and reproducible technique to be applied to clinical samples. The pairing of nucleotide bases facilitates the use of fragments of DNA that will hybridize to its complementary sequence. Probe labeling has facilitated their detection and in course of time colorimetric, fluorescent and chemiluminescent detection methods are replaced by the radioactive methods. In Southern blot, the genomic DNA is digested with a restriction enzyme, separated by gel electrophoresis and transferred to a membrane. The membrane is then treated with specially designed probes for the identification of target DNA (Garcia et al., 1998; Aries et al., 1998). It requires large quantity of DNA to confirm the identity of the amplified nucleic acid. Restriction fragment length polymorphism (RFLP) reveals the differences in sequences due to gain or loss of recognition sites for restriction endonuclease enzymes. Other methods such as Single-stranded conformation polymorphism (SSCP) (Spinardi *et al.*, 1991), denaturing gradient gel electrophoresis (DGGE) (Myers *et al.*, 1987; Fischer and Lerman, 1983) and RNase protection assay (RPA) (Winter *et al.*, 1995) can also demonstrate single nucleotide variations between fragments of nucleic acid of the same length. All these methods and application of probes can confirm the specificity of the PCR product. Sequencing provides the greatest level of detail in analysis of genetic material from a pathogen by exhibiting order of four bases in a fragment of DNA (Sanger *et al.*, 1977; Maxam and Gilbert, 1980). Its application in developing molecular methods for diagnosis and in epidemiology is highly commendable. Future innovation in hardware may bring sequencing in to the range of rapid and easy diagnostics like sequencing based on real-time pyrophosphate (Ronaghi *et al.*, 1998).

New Molecular Techniques

The ultimate aim of new tests is usually to improve the sensitivity and/ or specificity of diagnosis. The analysis of nucleotide sequences can provide much more detailed information on a pathogen than its phenotypic study. So, new molecular techniques have been developed for all economically important pathogens of aquaculture interest. Statistical comparisons of molecular and other methods of detecting aquatic pathogens have been reported for molluscan disease (Stokes *et al.*, 2002; Diggles *et al.*, 2003). These reports are extremely valuable, indicating the relative sensitivity and specificity of individual and combinations of tests. But, their wide application to the level of expectation is limited based on the satisfactory performance at field. Typing isolates by analysis of the small subunit, or 16S, ribosomal RNA genes appears to be a useful tool for the detection of *Streptococcus iniae* from human patients (Lau *et al.*, 2003). The addition of data from molecular diagnostics can assist epidemiological analysis of disease outbreaks and disease management. Molecular tests can provide better sensitivity or quantification, and techniques such as *in situ* hybridization allow assessment of the location of the pathogen within the tissues of the host. One of the most significant improvements in molecular diagnostics could be improvised for the control of pathogens in regional, national and international levels viz the detection of carrier fish or infected ova (Taksdal *et al.*, 2001; Brown *et al.*, 1994). The ability to diagnose a fish carrying sub clinical levels of pathogens is a great advantage. Carrier fishes need not be reared to maturity and they could be removed from a population to reduce the risk of spreading the pathogen . The ability to test fish through non-lethal sampling of mucus, blood or biopsy is a significant benefit offered by molecular methodology (Griffiths and Melville, 2000). Nucleic acid amplification can be combined with antibody binding to amplify a signal and improve sensitivity of detection (Schweitzer *et al.*, 2002). Other amplification methods like rolling circle amplification and real-time PCR (Lizardi *et al.*, 1998; Jordan 2000; Overturf *et al.*, 2001; Tang and Lightner 2001; DeFrancesco, 2003) may become more popular in disease diagnostics in near future. Many advances in methods and equipments are being developed and verified first in research before these are adopted for routine diagnostic use. Microarrays is one of such advanced methos which is mainly used in expression studies (Gerhold *et al.*, 1999; Dong *et al.*, 2001; Cheung *et al.*, 2002; Ganesan *et al.*, 2002) and are also being applied in proteomics (Zhu *et al.*, 2001; Haab *et al.*, 2001), despite the fact that there are some drawbacks (Knight, 2001). Microarrays will dramatically alter the speed and scale of molecular analysis of pathogens. The use of microarrays can assess many genes or polymorphisms (Jaccoud *et al.*, 2001) at once, providing a more detailed picture of the organism. Arrays are now being applied using antibody-antigen binding (de Wildt *et al.*, 2000) and these open up another avenue for analysis, alongside host-pathogen interactions (Cummings and Relman, 2000). The other major advance in the near future is likely to be the development of "laboratory-on-a-chip" devices that will enable on-site molecular detection and analysis. Further electronic chips could carry out amplification and hybridization (Kopp *et al.*, 1998; Umek *et al.*, 2001; Cheng *et al.*, 1998) and if these devices can be fabricated in a robust format, they could permit analysis on-site that currently requires several days and man-power in specialized laboratories.

Greater Sensitivity: Boon or Bane?

The application of molecular diagnostics is recently gaining acceptance and popularity. The ability to discriminate different strains of virus or bacteria provides new opportunities for controlling only harmful types. Equipments, reagents and practices vary between laboratories and may require modification before a test is performed satisfactorily. There have been many instances where a single protocol can perform in different ways in different laboratories. The fact that a single protocol may not suit all laboratories should not prohibit the application of different methods in different situations or locations. Indeed, application of more than one test may even instill greater confidence if the results concur. So, there is no substitute for practical experience with a method for instilling confidence in the technique and in the interpretation of its results. It should be remembered that not all PCR primer sets or methods will perform equally well. Henceforth, it warrants comparison of different methods to provide some validation. Conducting inter-laboratory or ring testing will be an excellent way of examining inter-laboratory or inter-test performance. During this process, great care must be taken to ensure that test materials sent to different laboratories are suitable and comparable; this is often the greatest challenge in setting up inter-laboratory comparisons. This will pave way for accreditation and quality assurance for the particular lab and its protocol.

On farm use of Molecular Techniques

A rapid diagnosis should be more vital for appropriate response and treatment for a fish and/or shrimp population than terrestrial animals. If culture of an organism is required, the time lapsed between the sampling and the results can allow spread of the pathogen to entire population. So, a swift identification is needed to preventing infection and total loss of production. Future developments in technology and methods are likely to provide probes, dipsticks, or hand-held thermocyclers that can be employed for an on-site diagnosis. Identification of the strain of pathogen can be important for treatment, which could be detected through plasmid analysis in a shorter time frame than conventional methods. This would enable appropriate and fast treatment to prevent losses of fish due to spread of the bacteria.

Bottlenecks in the Application of Molecular Diagnostics

There are some limiting factors that presently restrict the large-scale applications or throughput. Scaling up PCR, hybridization and sequencing has been possible through the use of 96-well formats to be economical. Phenol/chloroform-based methods for nucleic acid extraction perform well but are laborious and involve harmful chemicals. Various columns are produced commercially for extraction of DNA and/or RNA from a variety of starting material. If suitable, these kits can greatly improve the ease and efficiency of extraction. Before they are adopted, care must be taken to ensure that they provide equivalent yields of nucleic acid to other methods (Kok *et al.*, 2000) and to prevent cross-contamination, which is always a prime concern in clinical diagnostic testing. Gel

electrophoresis and other methods of analyzing nucleic acids can also restrict throughput. These problems have largely been overcome in systems such as real-time PCR which avoid the use of gels altogether. Initially, molecular tests were largely developed in laboratories that were devoted mainly to research. It needs closer cooperation of research and diagnostic groups to ease the transfer of technology at field level. The establishment of any diagnostic facility requires considerable investment in laboratories, equipment and trained personnel. The costs of maintaining such facilities are escalating as there is increased demand and pressure for these laboratories to be accredited and maintain Quality Assurance systems. Finally, as molecular diagnostics have gradually developed and increased in popularity, peer pressure will also influence decisions to adopt these techniques. It may be more economically viable to have centers of excellence for certain pathogens or techniques, and refer samples to these centers instead of having expensive equipments duplicated in various laboratories with a limited use. Concern over specificity of results is often a major obstacle for the adoption of PCR or probes for fish pathogens. It has been claimed that not enough is known about other organisms that may be present in the sample but are not pathogenic, yet may cross-react with primers or probes to yield a false positive result in a molecular test. So, thorough knowledge of the type of organism for which the test aims, together with information on the variability of the genome regions being targeted will guide an appropriate choice of primer or probe.

Molecular Therapeutics

An understanding and exploring the natural antiviral immune mechanism induced by dsRNA will bring a solid conceptual framework for the development of strategies to control viral diseases in shrimp aquaculture. The dsRNA-mediated posttranscriptional gene silencing [PTGS or RNA interference (RNAi)] has been described in both plants and animals including nematodes, *Caenorhabditis elegans* (Fire *et al.*, 1998), insects (Mirquitta and Paterson 1999) and mammals (Svoboda *et al*, 2000). Recently, it is proved in shrimp that *in vitro* synthesized dsRNA induces a general antiviral response. It may be either sequence-specific or sequence-independent (innate immunity) manner (Robalino *et al.*, 2004; Yodmuang *et al.*, 2006) as reported against three unrelated viruses such as White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV) and Yellow Head Virus (YHV) (Robalino *et al.*, 2005; Yodmuang *et al.*, 2006; Tirasophon *et al.*, 2005). The molecular basis for sequence independent immunity and the presence of Interferons (INFs) as in vertebrates remains elusive with the available knowledge on genomics and proteomics in crustaceans. Further, it is proved that siRNAs are less capable of inducing innate immune response than dsRNA in shrimp, *Litopenaeus vannamei* (Robalino *et al.*, 2005). So, it can be concluded that the product size of RNA plays a role in determining the anti-viral immunity. The ability of the cells, *in vivo*, to detect and internalize extracellular dsRNA to initiate intracellular gene silencing phenomenon implies the existence of cell surface receptors that mediate the uptake of dsRNA. It is found that dsRNA travels probably in the circulation from the site of injection to distant tissue and evinces highly sequence-specific gene silencing (Robalino *et al.*, 2007). It is not economically feasible to synthesize *in vitro* dsRNA and siRNA in large quantities for RNAi therapy in

shrimp culture ponds. As an alternative, production of bacterially expressed virus specific dsRNA will enhance the large-scale production of dsRNA for field application. Recently, Sarathi *et al*., (2008a) reported the maximum of 100 % survivability in *Penaeus monodon* against WSSV with intramuscular injection of bacterially expressed VP28dsRNA and 68 % survivability (Sarathi *et al*., 2008b) with oral administration of the same. It brings a need for further understanding of the first-pass effect in shrimp and can be explored to control viral genes with little or no risk of off-target effects as in pharmacological interventions. The ultimate identification of gene s responsible for RNAi will bring out exploration of this natural immunity in shrimp. But, question arises if RNAi is a natural antiviral immune mechanism, then at least some viruses should have evolved strategies to suppress or evade this phenomenon. Thus, it is proved that WSSV has more RNAi suppression than TSV as evidenced by loss of RNAi mediated down-regulation of STAT (Signal Transducer and Activator of Transcription) mRNA in WSSV infected shrimp (Robalino *et al*., 2007). So, it is important to understand the anti-RNAi functions of the virus particularly WSSV in shrimp.

CONCLUSION

The application of molecular diagnostics and therapeutics have incurred more intense scrutiny and calls for validation than any other methodologies. Perhaps, this is due to the difficulty of validating a test that is more sensitive than any other. The application of molecular methods in diagnostic testing offers ever-increasing advantages as further techniques and equipments are developed. Yet the adoption of molecular testing for fish and shellfish has, overall, been much slower than expected. Several factors will promote the use of molecular diagnostics and these should be encouraged wherever possible. Data from validation trials should be made available, if not via traditional publications, then at least through the incorporation on websites, etc. This will prevent any duplication and allow ready assessment of suitable methods to adopt for each particular circumstance. The use of parallel testing should not be underestimated and should readily be accepted as a means of validation for molecular diagnostics. After the advent of successful antiviral molecular therapeutics, appropriate drug delivery methods for complex and dynamic aquaculture system still provide challenges and needs further improvements. Finally, practical experience of molecular tests and their advantages in terms of sensitivity speed or detail of results should get wide publicity and encourage investment in the facilities itself to moniter fish and shellfish health. It is expected that further airing of optimistic approach in adoption of molecular diagnostics and therapeutics will promote advancement of this field overcoming the present bottlenecks.

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