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Molecular Tools for Monitoring Infectious Diseases in Aquaculture Species

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ABSTRACT

The rapid detection and identification of pathogens in infected fish, both clinically and subclinically, and in environmental samples is essential for effective health management in aquaculture. Traditional diagnostic techniques have been very useful for the identification of infectious agents in cultured fish and shellfish; however, they are limited in their speed and sensitivity. For this reason, the application of molecular biology techniques in this field has offered a wide range of possibilities in terms of decreased time required for diagnosis, and increased specificity, sensitivity and quantification of the infectious agent. This review summarises the most relevant molecular tools which have been used in aquaculture species.

Keywords: LAMP, microarrays, padlock amplification, PCR, RFLPs

Abbreviations: DNA, deoxyribonucleic acid; **ELISA**, enzyme-linked immunosorbent assay; **IHNV**, infectious hematopoietic necrosis virus; **IPNV**, infectious pancreatic necrosis virus; **ISAV**, infectious salmon anaemia virus; **LAMP**, loop-mediated isothermal amplification; **PCR**, polymerase chain reaction; **qPCR**, quantitative polymerase chain reaction; **RAPD**, randomly amplified polymorphic DNA; **RFLP**, restriction fragment length polymorphism; **RNA**, ribonucleic acid; **RSIV**, red seabream iridovirus; **RT-PCR**, reverse transcriptase polymerase chain reaction; **SVCV**, spring viraemia of carp virus; **VHSV**, viral haemorrhagic septicaemia virus

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INTRODUCTION

From the outset, the aquaculture industry has had to deal with numerous problems caused by different infectious agents, including viruses, bacteria, as well as protistan, myxozoan and crustacean parasites. Moreover, the increase in the number of farmed species during the last years has resulted in the appearance of new diseases. As a result of the external alterations, which cause a decrease in the commercial value, and the high mortality rates in farmed fish stocks, this sector has consistently been affected by important economic losses. Therefore, the effective prevention and treatment of infectious diseases is extremely important. However, despite the rising number of commercialised vaccines, only a few diseases can be controlled through prevention and, thus, the administration of an appropriate treatment remains the major way of fighting outbreaks. To that purpose, the availability of rapid diagnostic techniques is essential.

The clinical signs in fish infectious diseases are frequently non-specific and cannot be used for direct diagnosis. On the other hand, traditional diagnostic techniques based on the observation of cytopathic effects on cell cultures in the case of viruses, and the isolation and growth in specific culture media, specific biochemical and serological tests or *in situ* microscopic examination in the case of bacteria, sometimes lack speed or sensitivity, especially when working with certain microorganisms.

Considering all these facts, it can be concluded that sensitive, specific and fast diagnostic systems are needed. The recent advances in molecular biology have provided new methods, most of them PCR-based, which have led to significant improvements in both sensitivity and speed. Singleplex PCR has been extensively used for the development of diagnostic techniques for different fish and shellfish pathogens. More recently, this basic PCR has been modified to widen its utility and versatility. Thus, multiplex PCR allows the simultaneous detection of several pathogens in a single reaction. For example, this system has been successfully applied to the detection of *Yersinia ruckeri*, *Aeromonas salmonicida* and *Flavobacterium psychrophilum* in salmonids (del Cerro *et al.* 2002), as well as three different viruses

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(Williams *et al.* 1999). Other variants are nested PCR, a double amplification reaction that increases reaction sensitivity and specificity and is used to detect, for instance, *Renibacterium salmoninarum* (Chase and Pascho 1998); and reverse transcriptase PCR (RT-PCR) that enables the detection of RNA viruses like the infectious salmon anaemia virus (ISAV) (Devold *et al.* 2000; Mikalsen *et al.* 2001). The last generation of PCR techniques is quantitative (or real-time) PCR which allows the reduction of multiple, laborious and dangerous steps after the amplification process and, therefore, a decrease in the time needed for diagnosis. Real-time PCR techniques require fluorescent dyes and there are different alternatives, such as TaqMan, FRET and molecular beacon probes, to choose depending on the specific conditions of the assay. This system has already been applied for different fish pathogens with good results, such as the parasite *Myxobolus cerebralis* (Cavender *et al.* 2004; Kelley *et al.* 2006). Loop-mediated isothermal amplification (LAMP) and molecular padlock amplification are novel methods that reduce considerably the time required for detection. LAMP permits the rapid and specific amplification of DNA under isothermal conditions, and has been applied to the identification of several pathogens like *Edwardsiella tarda* (Savan *et al.* 2004). The molecular padlock amplification technique has been successfully used for the detection of the RNA viruses infectious hematopoietic necrosis virus (IHNV) and ISAV without a reverse transcription step (Millard *et al.* 2006). Finally, the development of DNA microarrays comes as a new means of simultaneous detection of several bacterial species. For instance, Warsen *et al.* (2004) recently developed a DNA microarray which permits the detection of 15 fish pathogens, such as *A. salmonicida*, *Edwardsiella ictaluri* and *F. psychrophilum* amongst others.

This review presents the latest advances on molecular diagnosis of fish pathogens, as well as discusses the future perspectives in this field.

CONVENTIONAL PCR APPROACH

This simple but extremely useful technique has definitely come as a revolution in the field of molecular biology. Among its different applications, one of the most important is, without a doubt, the field of infectious diseases, being a powerful tool for the identification of the causative agents.

By using a thermoresistant DNA polymerase, and primers corresponding to the flanks of a known DNA sequence, a series of cycles combining different temperatures and times leads to the amplification of a specific DNA fragment.

PCR has distinct advantages as a diagnostic tool over conventional microbiology, virology, parasitology and mycology. For instance, it is faster and more sensitive. It is especially appropriate for the detection of slow growing, difficult-to-cultivate or uncultivable pathogens as well as in situations in which inhibitory substances like antimicrobials are present. Furthermore, due to the stability of DNA, the detection can be performed from a wide variety of samples.

The principal shortcomings in applying PCR assays to the clinical setting include false-positive results from background DNA contamination; detection sensitivity exceeding clinical significance; and limited detection space of the assay or platform for simultaneous identification of multiple species, virulence factors or drug resistance.

Over the past two decades, PCR has been extensively modified to expand its utility and versatility as well as to improve automation and reproducibility.

The rapid detection of pathogens in infected fish and shellfish, both clinically and subclinically, is desirable for effective health management in aquaculture. In that sense, the utilisation of PCR is considered a helpful complement to conventional identification methods based on the isolation of the organism in the laboratory and its subsequent biochemical and phenotypic characterisation. The combination of both allows a faster and more efficient identification of the pathogen. However, PCR alone is not generally accepted as a detection procedure because of the need for interlaboratory validation and the putatively false positive and false negative results.

Conventional PCR-based techniques have been developed and tested for the detection of many fish and shellfish pathogens. For example, the bacterial pathogens *F. psychrophilum* (Urdaci *et al.* 1998; Cepeda and Santos 2000; Crumlish *et al.* 2007), *Flavobacterium columnare* (Bader *et al.* 2003; Suomalainen *et al.* 2006), and *Tenacibaculum maritimum* (Avendaño-Herrera *et al.* 2004; as well as the parasites *Microsporidium seriolae* (Bell *et al.* 1999), *Ichthyophonus hoferi* (Whipps *et al.* 2006), and *Cryptocaryon irritans* (Chen *et al.* 2008). It has also been used for the detection of viruses like the Pilchard herpesvirus (Crockford *et al.* 2005) and lymphocystis disease virus (Cano *et al.* 2007).

In most of these studies, the target DNA sequence belongs to the rRNA operon. In bacteria, the variable region of the evolutionarily conserved 16S rRNA gene is the most frequently used. In the case of *Piscirickettsia salmonis* the primers were designed based on alignment sequences of the internal transcribed spacer (ITS) and the flanking 23S rRNA gene of the ribosomal operon. This fragment presents variability within this species, which allows through sequencing or denaturant gel electrophoresis to document the presence of different and even new strains (Marshall *et al.* 1998; Heath *et al.* 2000). Nevertheless, other types of genes or even sequences of unknown function can be used. Examples of the latter are the works of Leon *et al.* (1994), who amplified a 149-bp DNA sequence unique to *R. salmoninarum*, and Argenton *et al.* (1996), who reported the use of a specific DNA fragment from *Y. ruckeri* identified by random amplified polymorphic DNA (RAPD). Several studies proposed the utilisation of genes related to virulence. For example, two primer combinations allowed the differentiation between a truncated form and the complete active form of the surface array protein coding gene (*vapA*) from *A. salmonicida* and, therefore, provided information on the potential virulence of a strain (Gustafson *et al.* 1992). The detection of *R. salmoninarum* was carried out by using the gene encoding the 57-kDa major surface antigen (MSA), associated with cell agglutination (McIntosh *et al.* 1996; Miriam *et al.* 1997). Coleman *et al.* (1996) identified the presence of *Vibrio vulnificus* through the PCR-amplification of the cytolysin-haemolysin gene. The *rpoN* gene coding for sigma factor 54, which has been involved in the regulation of virulence in some microorganisms, was used for the identification of *Listonella anguillarum* (Gonzalez *et al.* 2003). Other examples are the detection of *A. salmonicida* by amplification of the *fstA* ferric-siderophore (Beaz-Hidalgo *et al.* 2008), and the use of the gene *toxR* to detect *Vibrio parahaemolyticus* (Croci *et al.* 2007) and *Vibrio harveyi* (Pang *et al.* 2006). Mata *et al.* (2004a) detected *Streptococcus iniae* by using a metabolic gene, the lactate oxidase (*lctO*), which has only been found in a few microorganisms. The B subunit of the DNA-gyrase and the gene *amiB* (peptidoglycan hydrolase N-acetylmuramoyl-L-alanine amidase) were also used for the identification of *E. tarda* (Lan *et al.* 2008) and *L. anguillarum* (Hong *et al.* 2007), respectively.

In addition to allowing the identification of pathogens in *in vitro* cultures, PCR can be applied to tissue samples, thus permitting a much faster detection of the infectious agent. This can be decisive for the administration of an early treatment. Generally, DNA is prepared from the tissues and is subsequently used as a template in the PCR. The most appropriate tissues must be assayed in each case, as distribution of the pathogen within the host varies. In other cases, the whole animal is used, for example, eels and oysters for the detection of *V. vulnificus* (Coleman *et al.* 1996). Nevertheless, it is important to be able to use other less invasive modes of sampling. Good examples are the detection in blood (Zlotkin *et al.* 1998; Altinok *et al.* 2001; Gonzalez *et al.* 2003; Beaz-Hidalgo *et al.* 2008), serum (Marshall *et al.* 1998), ovarian fluid (Miriam *et al.* 1997), and mucus (Beaz-Hidalgo *et al.* 2008). The study by Beaz-Hidalgo *et al.* (2008) permitted the detection of *A. salmonicida* in carrier fish using non-destructive techniques. Also of impor-

tance are the storage and treatment of the tissues after sampling. For instance, in the case of *F. columnare*, it has been demonstrated that the freezing and thawing as well as prolonged enrichment cultivation of the samples weakened detection significantly (Suomalainen *et al.* 2006). Nevertheless, a recent study successfully identified *F. psychrophilum* using formalin-fixed, wax-embedded fish tissues (Crumlish *et al.* 2007).

It is very important that PCR allows the detection of low levels of the pathogen, especially in order to detect its presence in carrier fish and apply preventive measures. The sensitivity varies depending on the pathogen and the tissues used for the reaction; but it is, in most cases, in the range of 2-50 cells/mg of tissue. For example, the detection of *A. salmonicida* (Gustafson *et al.* 1992), *R. salmoninarum* (Leon *et al.* 1994) and *Y. ruckeri* (Gibello *et al.* 1999) in tissues like kidney and spleen gave sensitivities in the order of 10 colony forming units (CFU)/mg, 22 CFU/mg and 20 CFU/mg of tissue, respectively. For blood samples, the detection levels can vary drastically. Thus, Argenton *et al.* (1996) and Altinok *et al.* (2001) described the great difficulty of detecting *Y. ruckeri* in blood samples; whereas Zlotkin *et al.* (1998) could identify 4 CFU of *Lactococcus garvieae* per reaction using blood samples taken from naturally infected fish.

MULTIPLEX PCR

A large number of individual PCR assays would be necessary if single primer sets are used on a large number of samples, which can be a relatively costly and time consuming process. To overcome these shortcomings and also to increase the diagnostic capacity of PCR, a variant termed multiplex PCR has been described. In multiplex PCR more than one target sequence can be amplified from a sample by including more than one pair of primers in the reaction. Multiplex PCR has the potential to produce considerable savings of time and effort. Since its introduction, it has been successfully applied in the field of infectious diseases; the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi and parasites.

Nevertheless, the optimization of multiplex PCRs can pose several difficulties, including poor sensitivity or specifity and/or preferential amplification of certain specific targets (Polz *et al.* 1998). Thus, thorough evaluation and validation is essential. It is also important to indicate that the identification of a pathogen alone does not demonstrate its involvement in the disease process. Therefore, especially when multiple agents are detected, additional information such as clinical diagnosis and past history of infections in the fish farm need to be taken into account.

Multiplex PCR has not been widely applied to the detection of fish pathogens. The first work was developed by Brasher *et al.* (1998) for the detection of *Escherichia coli*, an indicator of fecal contamination, and the microbial pathogens, *Salmonella typhimurium*, *V. vulnificus*, *Vibrio cholerae*, and *V. parahaemolyticus*, in shellfish. Five pairs of oligonucleotides were used, each of them was found to support PCR amplifications of only its targeted gene. The sensitivity of detection for each of the microbial pathogens was $\leq 10^1$-10^2 cells following a “double” multiplex PCR amplification approach. In this case, amplified target genes in a multiplex PCR reaction were subjected to a colorimetric DNA-DNA hybridisation assay. This assay showed sensitivity of detection comparable to the agarose gel electrophoresis method. Moreover, the colorimetric assay avoided the use of hazardous materials inherent to conventional gel electrophoresis and radioactive-based hybridisation methods.

Osorio *et al.* (2000) used a multiplex PCR approach to discriminate between two subspecies of *Photobacterium damselae*. Two pairs of primers directed to internal regions of the rRNA and *ureC* genes were utilised to analyse a collection of *P. damselae* strains. With this procedure, one subspecies showed two amplification products while the other showed only one, indicating the absence of the urease gene in one subspecies. The PCR results were corroborated by dot blot hybridisation experiments. This constituted the first successful discrimination between two subspecies using a PCR procedure.

A multiplex PCR assay based on the 16 rRNA was developed for the simultaneous detection of three major fish pathogens: *F. psychrophilum*, *Y. ruckeri*, and *A. salmonicida* (del Cerro *et al.* 2002). They are the aetiological agents of cold water disease, enteric red mouth disease, and furunculosis, respectively. These pathogens are common worldwide and produce considerable economic losses in the fish farming industry. Thus, a rapid and effective diagnostic method is essential for the application of specific treatment against these diseases. The assay proved to be specific and as sensitive as each singleplex PCR assay, with detection limits in the range of 6, 0.6 and 27 CFU for *A. salmonicida*, *F. psychrophilum*, and *Y. ruckeri*, respectively. The multiplex PCR was useful for the detection of the bacteria in artificially infected fish as well as in fish farm outbreaks. This multiplex PCR system permits a specific, sensitive, reproducible, and rapid method for the routine laboratory diagnosis of infections produced by these three bacteria.

Multiplex PCR was also useful for the specific detection of the main pathogens involved in warm-water streptococcosis in fish (*S. iniae*, *Streptococcus difficilis*, *Streptococcus parauberis*, and *L. garvieae*) (Mata *et al.* 2004b). Each of the four pairs of oligonucleotide primers exclusively amplified the target gene of the specific microorganism. The sensitivity of the multiplex PCR for fish tissues was 5×10^3 cells/g for *S. iniae*, 1.2×10^4 cells/g for *S. difficilis*, 1×10^4 cells/g for *S. parauberis*, and 2.5×10^3 cells/g for *L. garvieae*. It was useful for the specific detection of the four species of bacteria not only in pure culture, but also in inoculated fish tissue homogenates and naturally infected fish. The existence of different types of *Streptococcus* species involved in the streptococcosis in fish emphasises the difficulties of definitive identification based on phenotypic traits alone. Therefore, definitive identification cannot be made without the support of genetic data. Thus, Baeck *et al.* (2006) developed a multiplex PCR method to simultaneously detect and confirm *S. parauberis* and *L. garvieae*. The multiplex PCR assay confirmed the results obtained with other methods and offered a rapid and sensitive technique able to identify, both biochemically and serologically, otherwise indistinguishable species.

Another study carried out by Panangala *et al.* (2007) detected three important pathogens in warm water aquaculture: *F. columnare*, *E. ictaluri* and *Aeromonas hydrophila*. This technique proved to work both with pure cultures and experimentally infected fish. The sensitivity threshold in tissues for the three bacteria ranged from 3.4×10^2 to 2.5×10^5 CFU/mg.

Sanjuán and Amaro (2007) developed a multiplex PCR assay for the detection of *V. vulnificus* biotype 2 and simultaneous discrimination of serovar E strains (potential zoonotic strains). It was successfully applied to both diagnosis and carrier detection in field samples.

In some cases, multiplex PCR has been used for the detection of virulence factors, thus revealing the presence of virulent strains. For example, several virulence factors associated with different species of *Aeromonas* (*A. hydrophila*, *A. sobria*, *A. caviae*, and *A. veronii*) were detected in water and diseased fish samples from a trout farm (Nam and Joh 2007), and haemolysin-producing strains of *L. anguillarum* were detected in clinical specimens in the study by Rodkhum *et al.* (2006). This latter assay indicates the presence of pathogenic strains from samples containing as little as 10 bacterial cells.

Multiplex PCR was also developed for the simultaneous detection of three different fish viruses: IPNV, IHNV, and VHSV (Williams *et al.* 1999). The current method for detection of fish viruses requires isolation of virus by inoculation of cell cultures with homogenates of tissue samples collected from a statistically significant portion of the population (Amos *et al.* 1989; OIE 2006; USFWS and AFS-FHS

2007). When a virus is isolated, identification of the virus is required. This is usually accomplished by neutralisation tests with specific polyclonal antisera, which can take 1 to 4 weeks, or by enzyme immunoassays with monoclonal antibodies or polyclonal antisera. The multiplex RT-PCR assay, which can be completed in 48 h or less, provides significant savings in time, cost, and materials in comparison to the antibody neutralisation tests or the separate, individual PCR assays presently used to identify viruses isolated in cell culture from clinical materials. The sensitivity levels of the multiplex RT-PCR assay were 100, 1, and 32.50% tissue culture infective doses for IPNV, IHNV, and VHSV, respectively (Williams *et al.* 1999). Confirmation of the sensitivity of the multiplex RT-PCR assay in field trials using tissue samples from naturally infected fish will provide a diagnostic assay with significant advantages over the current method of virus isolation in cell culture.

NESTED PCR

In this PCR-based method, two pairs of primers are used in two successive runs of polymerase chain reaction. The PCR product obtained in the first reaction is the template for the second reaction, in which the second set of primers bind within this product amplifying an amplicon shorter than the first one. This technique has higher specificity than single round PCR since it is quite unlikely that possible unspecific PCR products generated in the first round contain homolog sequences to the second pair of primers. Another advantage is that nested PCR requires less amount of target DNA due to the second amplification round and, as a result, it is more sensitive. On the other hand, the main limitations of this PCR-based assay is the higher risk of carryover of amplified DNA which produces cross contamination, and the increase of specificity may not be significant enough to justify the utilisation of a more complex and lengthier technique. Thus, the use of nested PCR would be recommended only for those cases in which the detection levels, in terms of either sensitivity or specificity, achieved with single-round PCR are not sufficient to identify the pathogen.

The nested PCR has been used to detect several bacterial fish pathogens. For example, Arias *et al.* (1995) developed a nested PCR method for detection and identification of *V. vulnificus* in eel farms, combining the use of universal and specific oligonucleotides directed against 23S rRNA in a two-step amplification. The detection limit of this method was 10 fg of *V. vulnificus* DNA, corresponding to one cell. In the case of *R. salmoninarum*, the PCR protocol described was based on the amplification of a sequence of the p57 gene (Chase and Pascho 1998). The nested PCR detected as few as 10 *R. salmoninarum* cells in salmonid kidney tissue, which means a 100-fold increase in sensitivity in comparison with conventional PCR. Osorio *et al.* (1999) described a method for the detection of *P. damselae* based on two highly variable regions of the 16S rRNA gene of the family *Vibrionaceae*. Three primers for a seminested PCR assay, as well as a short confirmatory DNA probe were used. The detection levels of this method were approximately 20 to 200 cells. These authors reported that this PCR-based protocol was at least 100 times more sensitive than a serological method based on a magnetic bead enzyme immunoassay. Nested PCR methods based on 16S rRNA primers (two universal eubacterial primers and two specific primers) have been also proposed for the detection of *F. psychrophilum* (Izumi and Wakabayashi 1997; Wiklund *et al.* 2000). Baliarda *et al.* (2002) developed an alternative nested PCR strategy based on 16S rRNA and 16S-23S rRNA intergenic spacer region primers to detect *F. psychrophilum* in biological samples (ovarian fluid, spleen, kidney and eggs). The level of sensitivity of this nested PCR was 0.25 CFU in contrast to 1.5 CFU described by Izumi and Wakabayashi (1997) or 0.4 CFU described by Wiklund *et al.* (2000). The latter author also detected 17 CFU/mg of brain tissue. In comparison, Baliarda *et al.* (2002) retrieved 2.5 CFU/mg of spleen being approximately 10 times more sensitive. More recently, Izumi *et al.* (2005) developed a nested PCR based on the *gyrB* gene, which allows the detection of low levels of *F. psychrophilum* in washings of fish gill surfaces and benthic diatoms as environmental samples. Bader *et al.* (2003) designed a set of species-specific 16S rRNA primers for detection of *F. columnare* in various tissues from live and dead fish. Nested PCR using universal eubacterial primers increased the sensitivity five-fold in comparison with the first PCR round, allowing the detection of *F. columnare* strains at DNA concentrations below 0.05 ng and from as few as 10 cells. Other two nested PCR systems were developed for detection of *T. maritimum* and whose detection limits were 75 CFU/mg fish tissue (Cepeda *et al.* 2003) and 1 to 250 cells per reaction depending on the tissue employed (Avendaño-Herrera *et al.* 2004). Finally, a nested PCR assay permitted the identification of the gene *gyrB* of *Pseudomonas plecoglossicida* in samples of kidney and intestine (Izumi *et al.* 2007).

Nested PCR assays were also used for detection of fish parasites. Bell *et al.* (1999) developed a nested PCR protocol for the detection of the microsporidian parasite *Microsporidium seriolae*, which affects farmed Japanese yellowtail. The nested PCR, capable of detecting as few as 0.01 pg of parasite DNA, was several orders of magnitude more sensitive than the standard single PCR, but was less specific limiting its utility. Barlough *et al.* (1995) were able to detect *Enterocytozoon salmonis* DNA from 10 infected chinook salmon lymphocytes using a nested PCR protocol. Mendonca and Arkush (2004) designed a set of species-specific 18S rRNA primers for detection of *Sphaerothecum destruens* in fish tissues. The limits of detection using the nested PCR test with these primers were 1 pg for purified parasite genomic DNA and 0.1 fg for plasmid DNA. Recently, Skirpstunas *et al.* (2006) used a modification of the nested PCR assay developed by Andree *et al.* (1998) to detect early stages of *M. cerebralis* in caudal and adipose fin samples from rainbow trout (*Oncorhynchus mykiis*). Although single round PCR assays allowed the identification of this microorganism in fish samples, the need for very invasive tissue sampling methods did not make it suitable for detection in live fish. The study by Skirpstunas *et al.* (2006), however, proved the effectiveness of a non-lethal sampling method coupled with nested PCR.

Two rounds of amplification of 529 and 379 bp fragments of the gene coding for the major capsid protein improved 10-fold the sensitivity in the detection of the Cyprinid herpesvirus-3 from tissues of infected fish. This PCR could also discriminate from the Cyprinid herpesvirus 1 and 2 (El-Matbouli *et al.* 2007).

REVERSE TRANSCRIPTASE PCR (RT-PCR)

This molecular technique allows the detection of RNA by converting this nucleic acid into a complementary DNA copy through the process of reverse transcription, and then amplifying using PCR. The target RNA sequence is transcribed using either random hexanucleotides or sequence-specific primers.

Most PCR methods detect DNA which persists in dead cells for significant periods of time (Masters *et al.* 1994). DNA was also demonstrated to persist in a PCR-detectable form in culture-negative environmental (Deere *et al.* 1996) and clinical samples (Hellyer *et al.* 1999). In contrast, RNA is known to be rapidly degraded with a typical half-life of minutes after cell death; thus, it has been proposed as a more accurate indicator of viable microorganisms (Alifano *et al.* 1994; Keer and Birch 2003). RT-PCR may help in differentiating viable from non-viable organisms, which is important for decisions in clinical practice. However, it is necessary to point out some limitations of this method. Firstly, detectable concentrations of intact RNA from a small number of cells are required to perform this technique. Moreover, the RNA sample must be free of genomic DNA, so special care must be taken during RNA isolation to avoid false-positive results.

There are few examples of RT-PCR based methods reported for detection of aquatic pathogenic bacteria. Magnusson *et al.* (1994) developed a nested RT-PCR assay based on the amplification of hypervariable regions within the 16S rRNA to detect *R. salmoninarum* in ovarian fluid of salmonid fish. The detection limit of this assay was one cell per sample. Cook and Lynch (1999) designed another nested RT-PCR method for detection of this pathogen; but, this time, amplifying mRNA from the gene encoding the antigen p57. Although the sensitivities of both methods were similar, the second technique was more reliable since mRNA is less stable than rRNA, thus reducing false positives due to the presence of non-viable bacteria.

RT-PCR methods are more widely used to detect RNA fish viruses, being an important alternative to cell culture protocols that are costly, lengthy and less sensitive. The most common genes detected are the nucleoprotein (N) and the glycoprotein (G). An RT-PCR method was developed by Cutrin *et al.* (2005) to detect IPNV in carrier turbot (*Scophthalmus maximus*). Miller *et al.* (1998) developed an RT-PCR assay for the detection and differentiation of VHSV and IHNV in organ samples from trout. The primers used in this method corresponded to highly conserved regions of the glycoprotein G-gene sequences of the 2 viruses. RT-PCR protocols were also developed to detect the ISAV. Devold *et al.* (2000) described an RT-PCR protocol for diagnosis of ISAV in carrier sea trout (*Salmo trutta*), and Mikalsen *et al.* (2001) used this technique to study the early phase of the virus infection in Atlantic salmon. RT-PCR diagnosis methods have been also used for diagnosis of other viral aquatic pathogens such as the sleeping disease virus (SDV) (Villoing *et al.* 2000), the spring viraemia of carp virus (SVCV) (Koutna *et al.* 2003), the walleye dermal sarcoma virus (WDSV) (Poulet *et al.* 1996), the extra small virus (XSV) (Sri Widada *et al.* 2004), *Macrobrachium rosenbergii* nodavirus (MrNV) (Sri Widada *et al.* 2003), the Taura syndrome virus (TSV) (Nunan *et al.* 1998), betanodaviruses (Gomez *et al.* 2004) and aquareovirus (Seng *et al.* 2004).

RT-PCR has also been combined with other molecular techniques such as RFLPs (López-Vázquez *et al.* 2006) and ELISA (Milne *et al.* 2006, 2007). In the first assay, a procedure was established for the detection of VHSV in trout by RT-PCR amplifying a region within the nucleoprotein gene. Milne *et al.* (2006) evaluated the ease of use, reliability and sensitivity of this method for the detection of IPNV in trout kidney samples. They could detect 1.5 x 10^4 plaque forming units (PFU). They concluded that this technique is safe, quick and reliable. The same laboratory used this colorimetric technique to detect human pathogenic viruses (enteroviruses and noroviruses) in bivalve molluscs (Milne *et al.* 2007).

The application of RT-PCR together with other types of PCR has allowed for improvements in the detection of certain pathogens. For example, a multiplex real-time RT-PCR permitted the simultaneous detection of three rhabdoviruses, SVCV, IHNV and VHSV, without non-specific amplification or cross-reactivity (Liu *et al.* 2008). More recently, RT-PCR has been combined with qPCR in order to detect and quantify pathogenic viruses. For example, Nerland *et al.* (2007) quantified nodaviruses in sea water at rearing facilities; Snow *et al.* (2006) detected the ISAV by means of semi-quantitative TaqMan real-time RT-PCR; Dhar *et al.* (2008) utilised SYBR Green to detect IHNV in liver, kidney, spleen, adipose tissue and pectoral fin samples from laboratory and field collected fish; and Bowers *et al.* (2008) monitored the presence of IPNV in pectoral fin, spleen and head kidney. In the last two studies quantification allowed the determination of different viral loads in these tissues throughout the infectious process, as well as time course assays. In some cases the high specificity and sensitivity allowed the use of non-lethal tissue sampling. There is also one study which describes the application of a real-time RT-PCR for the quantification of a pathogenic bacterium. Suzuki and Sakai (2007) detected the *msa* gene mRNA of *R. salmoninarum* in ovarian fluid and kidney samples from fish with clinical and subclinical infections.

REAL-TIME PCR

Real-time PCR is a technique designed to detect and quantify sequence-specific PCR products as they accumulate in 'real-time' during the amplification process. This system is based on the detection and quantitation of a fluorescent reporter, whose signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

There are two main fluorescence-monitoring systems for DNA amplification. The cheaper alternative is the use of DNA binding agents, non-sequence specific fluorescent intercalating agents, like SYBR-green I or ethidium bromide. This system quantitates amplicon production (including non-specific amplification and primer-dimer complex). The second and more popular alternative is the use of a fluorescent-labelled internal DNA probe which specifically anneals within the target amplification region. The choice of probe format depends on the compatibility of its hybridisation chemistry with the experimental design. Variations in probe format include TaqMan, fluorescence resonance energy transfer (FRET), scorpion probes, and molecular beacon probes.

The main advantages of using real-time PCR are: it is not influenced by non specific amplification (when specific probes are used), amplification can be monitored real-time, no post-PCR processing of products (low contamination risk), ultra-rapid cycling (30 minutes to 2 hours), wider dynamic range of up to 10^{10}-fold, requirement of 1000-fold less DNA or RNA than conventional assays (3 picogram = one genome equivalent), detection is possible down to a two-fold change, more specific, sensitive and reproducible. It also has some disadvantages; the most significant are that it is not ideal for multiplexing (amplifying multiple target sequences in one reaction by using multiple PCR primer pairs), setting up requires high technical skill and support, and high equipment and reagents cost.

Real-time PCR has enabled detection, identification and quantification of extremely low levels of aquatic pathogens. This is especially important for viral and parasitic diseases, in which diagnostic methods are more complex and require more time than bacterial pathogens. In fact, the rapid identification and quantification of viruses in diseased fish is a goal both conservationists and commercial aquaculturists have struggled to attain. There are many examples in which real-time PCR was successful in detecting viruses from infected fish and contaminated samples, proving to be as accurate as tissue culture assays yet much quicker. Utilising primers and fluorescent labelled probes generated for the specific identification of the nucleocapsid and glycoprotein genes of IHNV, the presence or absence of virus can be easily and rapidly confirmed. This method is also effective in measuring the relative or absolute quantity of virus present in the sample. This allows for the determination of the health status of carrier fish by measuring the quantity of viral genomes or transcribed viral genes present (Overturf *et al.* 2001). Similar real-time PCR assays allowed the identification and detection of many other viral pathogens, such as Iridovirus from large yellow croaker, *Pseudosciaena crocea*, using a molecular beacon (Wang *et al. 2006*); VHSV from rainbow trout (Chico *et al.* 2006), *Cyprinid herpesvirus 2* from moribund and healthy goldfish (Goodwin *et al.* 2006), and salmonid alpha-virus from salmonids (Hodneland and Endresen 2006). In this case, real-time PCR has allowed for the differentiation of several subtypes of this salmonid virus. These results demonstrated that the assay is

highly sensitive and specific (Hodneland and Endresen 2006). Starkey *et al.* (2006) developed a real-time nucleic acid sequence based amplification (NASBA) procedure, using a molecular beacon (carboxy-fluorecin [FAM]-labelled and methyl-red quenched) in order to detect ISAV. Amplification was carried out at 41°C for 90 min. This technique was 100 times more sensitive than conventional RT-PCR.

Apart from viruses, real-time PCR is also useful for the identification of important parasites such as *M. cerebralis*, a myxozoan parasite which causes whirling disease, a potentially lethal infection of salmonid fish. A TaqMan real-time quantitative PCR using the heat-shock protein 70 and 18S ribosomal DNA allowed the detection of this parasite in fish tissue, and provided a relative indication of infection severity (Cavender *et al.* 2004). If pathogens can also be detected and identified in the environment, it can be extremely useful as an early warning signal. To that end, a real-time PCR assay using SYBR Green intercalating fluorescent dye for rapid detection and quantification of *Ichthyophthirius multifiliis* has been developed. The assay can detect low concentrations of the parasite in water, presumably corresponding to an early phase of the disease (Jousson *et al.* 2005). The protistan parasite *Perkinsus marinus*, a pathogen of the oyster *Crassostrea virginica*, could also be detected and quantified in environmental water samples thanks to a SYBR Green-based real-time PCR at concentrations as low as the equivalent of 3.3×10^{-2} cells per 10 µl reaction mixture (Audemard *et al.* 2004). Real-time PCR assays also have been developed for rapid detection of *Pfiesteria piscicida. Pfiesteria* complex species are heterotrophic and mixotrophic dinoflagellates that have been recognised as a harmful algal bloom species associated with adverse fish and human health effects along the East Coast of North America. Until recently, specific identification of two toxic species known thus far, *P. piscicida* and *Pfiesteria shumwayae*, required scanning electron microscopy (SEM). SEM is a labour-intensive process in which only a small number of cells can be analysed, posing limitations when the method is applied to environmental estuarine water samples. To overcome these problems, a real-time PCR-assay that permits rapid and specific identification of these organisms in culture and heterogeneous environmental water samples was developed (Bowers *et al.* 2000). Hallett and Bartholomew (2006) developed an assay for the detection of the myxozoan parasite *Ceratomyxa shasta* in river water samples based on the amplification of 18S rRNA by a TaqMan PCR. The assay detected 1 spore in 1 l of river water and showed potential in replacing traditional sentinel fish studies for detection of the parasite. Inhibition of the assay in some river samples was overcome by reducing the template volume and including serum albumin. It was utilised to investigate the temporal and spatial distribution of *C. shasta* in the river. Phelps and Goodwin (2007) carried out the detection of the microsporidian *Ovipleistophora ovariae* in Cyprinid fish tissues. Early infections without spores or light infections with low number of spores are easily missed in microsporidians. The assay proved to be specific, since no cross-reaction with other related microsporidians was found. The assay detects reliably less than 10 genomic copies and 0.14 spores. Maximum sensitivity was achieved when sonication was used during the DNA purification step. McBeath *et al.* (2006) developed a TaqMan assay suitable for the detection of the crustacean parasites *Lepeophtheirus salmonis* and *Caligus elongatus*, generally referred to as sea lice, and is based on the amplification of the mitochondrial cytochrome oxidase I gene. This method permitted the species-specific identification of larvae of both parasites in plankton samples, thus complementing the complex and time-consuming microscopic method routinely used for identification.

Finally, real-time PCR has also allowed for the detection of many bacterial fish pathogens. Enteric septicaemia of catfish (ESC), caused by the gram-negative bacterium *E. ictaluri*, is the most prevalent disease affecting commercial catfish farms. Diagnostic methods currently used are unreliable at low levels of infection. A real-time PCR assay has been developed that enables the detection of as few as 2.5 cell-equivalents of *E. ictaluri* DNA in whole blood and other tissues in less time and with more accuracy and sensitivity than traditional diagnostic methods. Other examples report the detection and quantification of *R. salmoninarum* by Chase *et al.* (2006) and Powell *et al.* (2005). The latter was carried out for both DNA and RNA, thus permitting determination if the pathogen was transcriptionally active. One example of the high sensitivity of real-time PCR is shown in the studies performed with *V. parahaemolyticus.* A TaqMan PCR assay based on the *gyrase B* gene (*gyrB*) sequence was developed for quantitative detection of this pathogen in seafood. A total of 300 seafood samples were analysed and 78 (26%) of these samples were positive for *V. parahaemolyticus* using a conventional culture method and 97 (32.3%) using the real- time PCR assay. All culture-positive samples were PCR positive. However, 19 samples positive by PCR were culture negative (Cai *et al.* 2006). Finally, Balcázar *et al.* (2007) developed an assay for sensitive species-specific detection and quantification of *A. salmonicida* in fish tissue by using self-quenched fluorogenic primers.

RFLPs

Restriction fragment length polymorphism (RFLP) is a technique in which microorganisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two microorganisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another. The isolation of sufficient DNA for RFLP analysis is time-consuming and labour intensive. However, PCR can be used to amplify very small amounts of DNA to the levels required for RFLP analysis and consequently more samples can be analysed in a shorter time.

RFLP was used as typing technique in fish viruses. The evidence of genomic variations between IPNV strains was determined by restriction fragment profiles (Heppell *et al.* 1992). RFLP analysis was also proposed as a method for typing new isolates of aqua-birnaviruses due to its simplicity (Cutrin *et al.* 2004), and was shown to give similar results to other molecular techniques such as the ribonuclease protection assay or sequence analysis used for genotyping the fish rhabdovirus, VHSV (Einer-Jensen *et al.* 2005). The assay was able to distinguish between marine and continental isolates of VHSV.

Epidemiological studies of different fish pathogens as *Vibrio ordalii* (Pedersen *et al.* 1996a), *L. anguillarum* (Pedersen *et al.* 1996b), *R. salmoninarum* (Rhodes *et al.* 2000b), *F. psychrophilum* (Izumi *et al.* 2003), *Vibrio tapetis* (Le Chevalier *et al.* 2003), *S. iniae* (Eldar *et al.* 1999), *L. garvieae* (Eldar and Ghittino 1999; Eyngor *et al.* 2004) and *F. columnare* (Arias *et al.* 2004; Darwish and Ismaiel 2005) used RFLP among other techniques. It was useful to differentiate, to see similarities or to define new species (Lunder *et al.* 2000; Martinez-Murcia *et al.* 2005; Panangala *et al.* 2005), to determine a group of isolates as unique (Rhodes *et al.* 2005), to differentiate (van der Sar *et al.* 2004) or to identify new strains (Bisharat *et al.* 1999). Nevertheless, there are some cases where RFLPs are not useful. For instance, 16S rDNA RFLP discriminated between *S. iniae* and other fish pathogens but not between *S. iniae* strains (Eldar *et al.* 1997). This method also suggested many hypotheses about clonal relationship and behaviour between the aquaculture and human compartments (Rhodes *et al.* 2000a; Giraud *et al.* 2004; Ucko and Colorni 2005). Izumi *et al.* (2007) identified genotypes associated with quinolone resistance by PCR-RFLP, which showed a different Mph1103I restriction pattern in the gene encoding the A subunit of the DNA gyrase.

RFLP and PCR-RFLP have been used in the direct identification and diagnosis of some fish pathogenic bacteria, by itself or combined with other techniques (Tiirola *et al.* 2002; Kozinska *et al.* 2002; Nilsson and Strom 2002; Pate *et al.* 2005; Zappulli *et al.* 2005). Moreover, RFLP can be useful to monitor the levels and species composition of bacteria in fish farms (Lee *et al.* 2002).

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

The loop-mediated isothermal amplification (LAMP) reaction is a novel method that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. The cycling reaction continues with accumulation of 10^9 copies of target in less than one hour. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity. LAMP has a wide-ranging applicability in the rapid detection of pathogenic microorganisms. Although it is still a novel procedure, it is very promising because of the lack of need of expensive equipment, a water bath would be sufficient to carry out the reaction, and its speed. However, this technique is not useful for the simultaneous detection of different pathogens.

Savan *et al.* (2004) first reported the application of LAMP for the diagnosis of a fish pathogen, *E. tarda*, in samples from kidney and spleen, as well as sea water. The conditions of the reaction were 45 min at 65°C. LAMP methods were also developed for other bacterial pathogens. For example, *E. ictaluri* (Yeh *et al.* 2005), *F. columnare* (Yeh *et al.* 2006), *Nocardia seriolae* (Itano *et al.* 2006), and *Y. ruckeri* (Saleh *et al.* 2008).

This technique has also been applied to viral pathogens, such as SVCV (Shivappa *et al.* 2008), VHSV (Soliman *et al.* 2006), IHNV (Gunimaladevi *et al.* 2005), koi herpes virus (KHV) (Gunimaladevi *et al.* 2004) and red seabream iridovirus (RSIV) (Caipang *et al.* 2004); and to parasites like *M. cerebralis* in fish and oligochaetes (El-Matbouli and Soliman 2005a) and another myxozoan parasite of salmonid fish *Tetracapsuloides bryosalmonae* (El-Matbouli and Soliman 2005b). The sensitivity levels obtained with this technique were always similar in the initial *in vitro* tests and in the *in vivo* experiments, both with experimentally infected fish and clinical samples.

In all these cases the incubation temperature was 63–65°C and the incubation time was about 60 min. Many of these studies describe the use of SYBR Green I stain for visual inspection instead of agarose gel electrophoresis with the same efficiency. In the study by Caipang *et al.* (2004) the synthesis of a large amount of DNA led to the production of a white precipitate, magnesium pyrophosphate, as a by-product. The presence or absence of this white precipitate facilitates easy detection of the RSIV genomic DNA without the use of gel electrophoresis. A strong correlation exists between the amount of input viral DNA copy and the corresponding turbidity reading at the end of the reaction; hence, the LAMP reaction may be used potentially to quantify RSIV particles in the infected fish.

MOLECULAR PADLOCK AMPLIFICATION

Millard *et al.* (2006) developed a new method for the molecular detection of IHNV and ISAV, which combines molecular padlock probe (MPP) technology with rolling circle amplification (RCA) and hyperbranching (Hbr). This technique allows the detection of RNA target sequence from these viruses at levels similar to those detected by PCR, but without a prior reverse transcription step. Thus, while exhibiting selectivity levels comparable with those of PCR, it could potentially reduce the time and cost required for the analysis. This method detected as few as 10^4 DNA oligonucleotide targets and was sequence-specific at the single base level. Viral RNA could be detected directly, either alone or in the presence of non-viral RNA from fish tissue. Interestingly, the authors suggest the possibility of integrating this technique into a biosensor platform, which could be used for on-site diagnosis of fish pathogens.

MICROARRAYS

Multiplex PCR allows the detection of several pathogens in the same reaction. The number of infectious agents that could potentially be identified simultaneously with this method is, however, considered to be a maximum of six. The limits are set by the necessity of optimizing the PCR conditions to use different pairs of primers as well as the challenge of differentiating the amplified products by gel electrophoresis. In fact, further analysis, such as DNA sequencing, is sometimes required to confirm product identity.

The use of DNA microarrays could overcome these limitations and permit the identification in one single step of a wide range of pathogens from complex samples. The products obtained in PCR would be, in this case, subsequently hybridised with a large number of probes. Furthermore, unlike conventional multiplex PCR assays, microarray detectors do not require clear length differences between PCR products. Therefore, PCR assays can be designed to generate small similar-sized amplicons, which would maximise efficiency and probability of template recovery from degraded DNA, as well as reduce PCR template bias.

Call *et al.* (2003) described the development of an array for the detection of 8 different fish pathogens (*R. salmoninarum*, *A. salmonicida*, *E. ictaluri*, *F. columnare*, *F. psychrophilum*, *Y. ruckeri*, *P. salmonis*, and *Tenacibaculum maritimum*). PCR was used in an initial amplification step and the products were then hybridised to the array to identify the species present in the sample by using 16S rDNA probes, suitable for distinguishing between several salmonid pathogens.

Gonzalez *et al.* (2004) coupled multiplex PCR and a DNA microarray to construct an assay suitable for the simultaneous detection of five important marine fish pathogens (*V. vulnificus*, *L. anguillarum*, *P. damselae* subsp. *damselae*, *A. salmonicida* subsp. *salmonicida*, and *V. parahaemolyticus*). The array was composed of nine short oligonucleotide probes complementary to seven chromosomal loci and two plasmid-borne loci. Nine primer sets were designed to amplify short fragments of these loci in a multiplex PCR. PCR products were subsequently labelled by nick translation and hybridised to the microarray. Using purified genomic DNA, PCR products could be detected with four or five cells, and the array was at least four-fold more sensitive than agarose gel electrophoresis for detection of PCR products.

More recently, a DNA microarray suitable for the simultaneous detection of 15 fish pathogens based on 16S ribosomal DNA polymorphisms was developed by Warsen *et al.* (2004). PCR products of a small size (ca. 199 bp) were generated using biotinylated, universal primer sequences, and these products were hybridised overnight to the microarray. The detection sensitivity for purified control DNA was equivalent to < 675 fg, and this sensitivity was not adversely impacted either by the presence of competing bacterial DNA or by the addition of fish DNA.

Although all these studies are carried out with *in vitro* bacterial samples, there is no doubt that the application of microarrays to tissue and environmental samples will open new possibilities in the field of detection of pathogens in aquaculture, by allowing to test the presence of a large number of species in a single assay.

CONCLUSION AND FUTURE PERSPECTIVES

The existence of adequate systems for the detection and identification of pathogens in farmed and wild fish is critical for economic and environmental benefits. Moreover, if

fast and effective techniques are available, this could prevent food products from fish or molluscs contaminated with human pathogens being marketed.

For a long time, people involved in the aquaculture industry realized that faster and more specific diagnostic techniques were necessary. Once molecular techniques started to be used in research concerning fish and shellfish pathogens, for instance to study the pathogenicity mechanisms, antibiotic resistance, etc., it became apparent that there was potential for their utilisation in the clinical field.

The development of molecular diagnostic methods will be very important to design new and more effective treatment programmes and methods of prevention, such as early detection in carriers or environmental samples. The information provided by these techniques could also be helpful for planning more specific vaccination programmes by taking into account the species or even strains which have been affecting a particular fish farm.

Nowadays, many of the techniques described in this review are being already applied for the detection and identification of pathogens in aquaculture diagnosis laboratories. The main difficulty in these methods becoming completely accepted is the necessity for appropriate standardisation and validation.

It is to be expected that in the future molecular techniques will be widely accepted for diagnosis in aquaculture and even methods like microarrays will be routinely used in laboratories, thus facilitating enormously an early identification of the causative agent. However, it is important to be cautious with the results obtained by these methods of molecular diagnosis. The mere detection of the presence of a pathogen in fish or shellfish samples from an outbreak does not necessarily imply that they are responsible for or even involved in the disease. This is of particular relevance in the case of techniques such as multiplex PCR and microarrays, which allow the simultaneous identification of several pathogens. Therefore, it is recommendable to take into account additional information, like clinical diagnosis, in order to determine the most appropriate treatment. Even more specific traits, such as antibiotic resistance gene profile or virulence genes could be identified and considered when deciding on the most adequate treatment. All of these measures will diminish the economic losses associated with infectious diseases, as well as the risk of infecting wild fish species. It will also permit the production of farmed fish and shellfish that will be safer for human consumption, by controlling the presence of human pathogens and by reducing the administration of antibiotics.

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