The Open Access Israeli Journal of Aquaculture – Bamidgeh

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The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB),
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and
University of Hawaii Aquaculture Program in association with
AquacultureHub
http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:
Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

Detection and Identification of Fish Pathogens: What is the Future?

A Review

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(Received 1.8.08, Accepted 20.8.08)

Key words: biosecurity, diagnosis, DNA array, multiplexing, real-time PCR

Abstract

Fish diseases pose a universal threat to the ornamental fish industry, aquaculture, and public health. They can be caused by many organisms, including bacteria, fungi, viruses, and protozoa. The lack of rapid, accurate, and reliable means of detecting and identifying fish pathogens is one of the main limitations in fish pathogen diagnosis and disease management and has triggered the search for alternative diagnostic techniques. In this regard, the advent of molecular biology, especially polymerase chain reaction (PCR), provides alternative means for detecting and identifying fish pathogens. Many techniques have been developed, each requiring its own protocol, equipment, and expertise. A major challenge at the moment is the development of multiplex assays that allow accurate detection, identification, and quantification of multiple pathogens in a single assay, even if they belong to different superkingdoms. In this review, recent advances in molecular fish pathogen diagnosis are discussed with an emphasis on nucleic acid-based detection and identification techniques. Major features and applications of current predominant methods and promising methods likely to impact future fish disease control and prevention are outlined.

Introduction

Diseases caused by fish pathogens, including bacteria, fungi, viruses, and protozoa, can cause considerable economic losses to the ornamental fish and aquaculture industries. Biosecurity (pathogen preventing) programs that address aquatic animal pathogens and diseases are becoming an increasingly important focus of these industries (Scarfe et al., 2006). Nevertheless, the lack of rapid, accurate, and reliable means by which fish

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pathogens can be timely detected and identified has been one of the main limitations in fish pathogen diagnosis, fish disease management, and biosecurity policies.

Conventional diagnosis methods often rely on interpretation of clinical and histological signs, culturing pathogens in or on a suitable medium, and analysis of morphological, phenotypic, or biochemical characteristics of the presumptive pathogen. Although these methods are fundamental to the development of any alternative diagnostic method, the accuracy and reliability of these techniques largely depend on competent (taxonomical) expertise. Further, diagnosis requiring a culturing step is generally time-consuming and labor intensive. For example, assays for *Flavobacterium* or *Mycobacterium* species may require several days for growth with specialized media and growth conditions (Nematollahi et al., 2003; Van Trappen et al., 2003). Detection of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonids, can take up to 12 weeks (Benediktsdottir et al., 1991). Moreover, these techniques rely on the ability of the organism to be cultured *in vitro*. This aspect considerably limits the applicability of these methods since possibly less than 1% of the microorganisms in an environmental sample may be cultured (Rappe and Giovannoni, 2003). Viruses are usually detected by designated virology laboratories using isolation, electron microscopy, *in vitro* viral culture or, if available, serological assays to detect viral antigens or test for the immune response to a given virus (Leong, 1995; Lightner and Redman, 1998; Storch, 2000).

Indeed, disadvantages associated with traditional identification techniques have triggered the search for alternative culture-independent detection and identification techniques, such as those based on the detection of antigenic determinants (serological techniques) or nucleic acids (nucleic acid-based techniques). Compared to traditional assays, these molecular techniques can avoid problems in investigating organisms for which no culture medium, cell lines (for viruses), or detection method is available. In addition, these techniques are generally faster, more specific, more sensitive, and more accurate (Cunningham, 2002).

One of the most common serological identification techniques is the enzyme-linked immunosorbent assay (ELISA; Clark and Adams, 1977) and its variations, which are all based on the binding between diagnostic antibodies and specific antigens of the target. Because of their versatility, simplicity, speed, and possibility to quantify the target pathogen, ELISA assays have been frequently used in pathogen diagnosis, especially for the detection of viruses and bacteria (Martinez-Govea et al., 2001; Wagner et al., 2001; Adkison et al., 2005; Reschova et al., 2007). Highly specific assays can be developed using monoclonal antibodies that recognize a specific epitope of the pathogen. However, to detect the different strains of a given virus, for example, polyclonal antibodies that target multiple epitopes of the pathogen are needed. Nevertheless, major limitations for the development of serological assays include that the required antiserum for detection of a pathogen be accessible and affordable and that the required degree of sensitivity and specificity is often difficult to reach (Adkison et al., 2005).

On the other hand, nucleic acid-based techniques, especially if they make use of polymerase chain reaction (PCR; Mullis and Faloona, 1987), have the advantage of being exceedingly sensitive and specific and requiring reagents that are easily available. As a result, PCR-based techniques have increasingly been developed for (fish) pathogen diagnosis (Cunningham, 2002). This trend is stimulated by the continuously growing availability of sequence data in databases such as GenBank (http://www.ncbi.nlm.nih.gov/Genbank/; Benson et al., 2004) and the increasing availability of microbial full genome sequences (e.g., http://www.sanger.ac.uk/Projects/Microbes/).

Nevertheless, although most of these methods are convenient for the detection of a single pathogen, screening for large numbers of different pathogens relies on a significant number of parallel tests, often using different technologies (Evangelopoulos et al., 2001; Lievens et al., 2005a). Consequently, testing

multiple targets using these methods is inefficient, laborious, and expensive. Therefore, a number of methods have recently been developed that can be used for the simultaneous detection of multiple pathogens, encompassing multiplex PCR, DNA arrays, and PCR arrays (Elnifro et al., 2000; del Cerro et al., 2002; Wang et al., 2002, 2003; Gonzalez et al., 2004; Mata et al., 2004; Warsen et al., 2004; Lievens et al., 2005a).

In this manuscript some recent advances in fish pathogen diagnosis are described, with an emphasis on nucleic acid-based detection and identification of the two major fish pathogenic groups for which classical detection may be problematic: bacteria and viruses. Major features and applications of the most predominant methods used nowadays and some methods that look promising for the future are outlined.

Nucleic Acid-Based Identification of Fish Pathogens

Choice of target sequences. The first stage in the development of nucleic acid-based diagnostic assays is the selection of specific sequences that can be used to identify pathogens. There are some generally applicable techniques for bacteria and fungi, but viruses usually need different approaches. Regarding bacteria, the most common strategy for selecting target sequences involves the use of ubiquitously conserved genes that harbor specific sequences. At present, the bacterial ribosomal RNA (rRNA) operon, encompassing a 16S rRNA and 23S rRNA gene as well as an intergenic spacer (IGS) region, is most frequently used in the development of molecular bacterial diagnostics (Ludwig and Schleifer, 1994; Call et al., 2003; Sachse, 2004; Toranzo et al., 2005). In particular, the 16S rRNA gene is commonly targeted (Drancourt et al., 2000; Hongoh et al., 2003; Warsen et al., 2004; Osborne et al., 2005).

There are several reasons why ribosomal sequences are such widely used targets for diagnostic development, including (a) its universal abundance, (b) its evolutionary and phylogenetic properties, reflected by the presence of both variable and highly conserved sequence domains, (c) its high discriminatory potential over a wide range of taxonomical levels, (d) its, often, multiple-copy nature, resulting in more sensitive analyses, and (e) the extensive availability of ribosomal DNA sequences in public databases. These extensive sequence data allow comparison of sequences and, in turn, determination of diagnostic regions that can be used to design specific primers, probes, or oligonucleotides. Nevertheless, ribosomal DNA sequences do not always reflect sufficient variation to discern particular species (Mollet et al., 1997; Blackwood et al., 2000; Thompson et al., 2004; Kupfer et al., 2006). Therefore, other housekeeping genes showing intertaxa sequence variation are becoming more intensively studied, including the DNA gyrase subunit B gene (*gyrB*; Watanabe et al., 2001; Yanez et al., 2003), genes encoding the RNA polymerase subunits A and B (*rpoA* and *rpoB*; Dahllof et al., 2000; Thompson et al., 2005; Tarr et al., 2007), the recombinase subunit A gene (*recA*; Thompson et al., 2004), genes encoding heat shock proteins (*hsp60, hsp65* and *dnaJ*; Nhung et al., 2007), the elongation factor-Tu encoding (*tuf*) gene (Mignard and Flandrois, 2007), and the gene encoding a manganese-dependent enzyme (*sodA*; Adekambi and Drancourt, 2004). However, in comparison with ribosomal DNA sequences, databases for these gene sequences generally contain a small number of sequences, necessitating extensive experimental screening to ensure specificity of a diagnostic assay based on these genes.

In contrast to bacteria, viruses may contain a DNA- or RNA-based genome, representing DNA or RNA [single (ss) or double stranded (ds)] viruses, respectively. In general, viral genomes are relatively small and in many cases data on complete virus genomes are available in sequence databases. Currently, one of the most common targets for virus diagnostics is the coat protein gene, but other regions such as the DNA or RNA polymerase gene are also being used (Culley et al., 2003; Ishioka et al., 2005). In fact, any part of the genome could be suitable depending on how much sequence data is available from

target and related virus strains in the same region of the genome. Nevertheless, since viral genomes, especially those of RNA-viruses, are prone to mutation, there are many groups of viruses for which no conserved sequences are available that can be used for the design of genus- or group-specific primers or probes. As a result, detection of emerging or uncharacterized viruses remains a great challenge in molecular virology.

Nucleic acid-based detection techniques can be divided into DNA- and RNA-based techniques; some of the most common are addressed below. Efficient extraction protocols and commercially available extraction kits are available for both types of genetic material, rendering highly purified DNA or RNA from biological samples such as water or fish tissue (e.g., Filter Service S.A., Eupen, Belgium; Mo Bio Laboratories, Solana Beach, CA, USA; Qiagen, Inc., Valencia, CA, USA; Fahle and Fischer, 2000), favoring their use in scientific research as well as routine diagnosis.

Polymerase Chain Reaction (PCR). Using PCR, millions of copies of specific DNA sequences are generated in a thermocyclic process consisting of repetitive cycles of DNA denaturation, primer annealing, and elongation using a thermostable DNA polymerase (Mullis and Faloona, 1987). If a DNA sequence unique to a particular organism is determined, specific PCR primers can be designed that allow determination of the presence or absence of that sequence and, thus, of the corresponding organism. The presence of amplified DNA is traditionally detected by gel electrophoresis, but alternative detection methods exist as well (e.g., Mutasa et al., 1996; Fraaije et al., 1999). Any pathogen having a DNA genome can potentially be detected in this way.

In addition, by inclusion of a step employing the reverse transcriptase enzyme, RNA targets such as RNA viruses can also be detected. This technique is referred to as reverse transcriptase PCR (RT-PCR; Raineri et al., 1991; Tan and Weis, 1992). Typically, RT-PCR consists of an annealing step for a reverse primer or a mixture of random primers and an extension step to synthesize a complementary DNA strand (cDNA), followed by a (real-time) PCR assay. Many reports describe the development of specific (RT-) PCR assays in the ornamental fish industry and aquaculture (Cunningham, 2002; Toranzo et al., 2005).

Clinical laboratories are increasingly using PCR to complement or replace classic diagnostic assays, often in the context of biosecurity (preventing) programs or to ensure the identity of a pathogen. Several PCR assays (Gibello et al., 1999; LeJeune and Rurangirwa, 2000; Altinok et al., 2001) have been developed for the specific detection of *Yersinia ruckeri*, the causative agent of enteric redmouth disease (ERM) or yersiniosis that can cause high morbidity and mortality rates in fish farms. To minimize economic losses, rapid, specific, and sensitive detection of this pathogen is needed and can be met by PCR. Spring viraemia of carp virus (SVCV) is an RNA virus responsible for a severe hemorrhagic disease in farmed cyprinids. Fast and timely detection of the virus is necessary because currently no vaccine against SVCV is commercially available. To avoid transmission of the virus, a specific RT-PCR assay employing a nested PCR has been successfully used to identify SVCV in fish tissue (Oreshkova et al., 1999; Koutna et al., 2003; Sanders et al., 2003). Another contagious viral disease is caused by koi herpes virus (KHV), a DNA virus responsible for significant morbidity and massive mortality in common carp (*Cyprinus carpio*), koi carp (*C. carpio koi*), and *C. carpio gio*. Because of its huge impact on the ornamental fish industry, increasingly sensitive detection techniques like PCR are being developed to detect this virus in an early stage of infection (Gilad et al., 2002, 2004; Bercovier et al., 2005; Ishioka et al., 2005; El-Matbouli et al., 2007; Matsui et al., 2008).

Depending on the primers and the detection method used, minute quantities of pathogen DNA can generally be detected using PCR (Gibello et al., 1999; Gilad et al., 2002; Bader et al., 2003). Nevertheless, to increase sensitivity (and specificity) nested PCR or immunocapture PCR (IC-PCR) may be used. Nested PCR involves two sets of

primers, used in two successive PCR reactions. The second reaction uses primers that hybridize to a sequence within the DNA fragment that is generated in the first reaction (Arias et al., 1995; Alonso, 1999; Wiklund et al., 2000; Welker et al., 2005).

IC-PCR makes use of immobilized antibodies to isolate the target pathogen from a sample prior to PCR amplification (Sharman et al., 2000; Peng et al., 2002). Alternatively, specific probes may be used to improve sensitivity and specificity (Greisen et al., 1994; Leon et al., 1994). The advantage of the higher sensitivity obtained by these methods can be exemplified for one of the most important pathogens in salmonid aquaculture, namely *Flavobacterium psychrophilum*, the causative agent of the rainbow trout fry syndrome and bacterial cold-water disease. Although Urdaci et al. (1998) had developed a specific PCR for this bacterium, the detection limit of the assay appeared too low to detect the pathogen at low densities, resulting in “false negatives” for subclinical or covert infections (Cipriano and Holt, 2005). Since even low pathogen concentrations can lead to considerable economic losses, more sensitive, nested PCR assays have been developed, enabling detection of this pathogen at low densities in fish tissue and water samples (Wiklund et al., 2000; Baliarda et al., 2002; Izumi et al., 2005; Madsen et al., 2005; Crumlish et al., 2007).

PCR is also used to quantify the amount of pathogen DNA. Although it is relatively easy to quantify the amount of PCR products, it is more difficult to relate this quantity to the original amount of target DNA. For certain pathogens, however, this information may be necessary to make suitable disease management decisions and for monitoring the effects of these decisions. Though challenging, pathogen DNA may be quantified using competitive PCR (Siebert and Larrick, 1992), which involves co-amplification of the target DNA and known quantities of competitor DNA amplifiable by the same primer pair that yield a product of a different length. The amount of initial target DNA is then determined on agarose gel by comparison of the relative amounts of target and competitor PCR products. This method has successfully been used to quantify, for instance, the bacterium *Piscirickettsia salmonis*, the causative agent of Piscirickettsiosis (Heath et al., 2000). However, designing an appropriate competitor might be problematic to ensure accurate DNA quantification. In addition, one should be careful to ensure detection at the exponential phase of the PCR reaction.

Quantitative real-time PCR. More recently, quantitative real-time PCR (Heid et al., 1996) has been proven to be reliable with regard to both pathogen detection and quantification. This technology is more sensitive, more accurate, and less time-consuming than conventional end-point PCR because it monitors PCR products as they accumulate during the reaction. This allows template quantification during the exponential phase of the reaction, before reaction components become limiting. In addition, since there is no need to open the tubes in which the amplification takes place, the likelihood of post-PCR carry-over contamination is greatly reduced. Typically, DNA amplification is monitored each cycle based on the excitation of fluorescent dyes and detection of fluorescent emissions (Heid et al., 1996; Mackay et al., 2002). In general, the initial amount of target DNA is related to a threshold cycle, which is defined as the cycle number at which fluorescence significantly increases above the background level. Target DNA is quantified using a calibration curve that relates threshold cycles to a specific amount of template DNA.

As extensively discussed in other reviews (Mackay et al., 2002; Hanna et al., 2005; Lievens et al., 2005a; Espy et al., 2006), accumulating amplicons can be detected using either amplicon specific or non-specific detection methods, i.e., sequence-specific probes or DNA-intercalating dyes, respectively. The use of a DNA-binding dye like SYBR® Green is more straightforward and less expensive then using probes but is less specific since the fluorogenic molecule binds to all double stranded DNA (dsDNA) present in the sample. Further, interpretation of the analysis may be disturbed by the formation of primer-dimers or aspecific PCR products. However,

the risk of the latter can be reduced by using highly specific primers and stringent reaction conditions and the accuracy (specificity) of the reaction can be checked by a melt curve analysis at the end of the PCR run (Bustin, 2000; Mackay et al., 2002).

In contrast to amplicon non-specific detection chemistries, probe-based assays like those based on a TaqMan® probe offer the advantage of increased specificity, certainly in combination with specific primers (Livak et al., 1995). These probes consist of a single stranded short oligonucleotide labeled with a reporter fluorophore at the 5' end and a fluorogenic quencher at the 3' end. Because of the proximity of both groups, the fluorescent signal is quenched. During the annealing phase of each PCR cycle the probe hybridizes to a specific region within the amplified fragment. During the elongation phase, the probe is degraded by the 5'-exonuclease activity of the DNA polymerase causing the release of the reporter from the quencher, resulting in a fluorescent signal.

A drawback for this technique is that no melting curve analysis can be performed and thus, in theory, false positive results can be obtained. This potential limitation may be circumvented by the use of a quenching probe (QProbe). A QProbe contains cytosine at its 5' or 3' end which is labeled with a guanine quench fluorophore. When a QProbe hybridizes with a target sequence, its fluorescence is quenched by the guanine in the target that is complementary to the modified cytosine. Consequently, in contrast to a TaqMan® probe-based assay, a reduction in fluorescence is measured as the PCR products accumulate during the reaction. In addition, no DNA polymerase is needed to obtain fluorescence. As a result, a DNA polymerase without 5'-exonclease activity can be used, which, in turn, enables the formation of a melting curve, allowing a specificity check of the reaction (Kurata et al., 2001).

Depending on the target gene selected, closely related microbial species may differ in only a single (or few) base(s) of the investigated gene. The high degree of specificity of real-time PCR technology allows detection of single nucleotide polymorphisms (SNP), independent of the detection chemistry (Livak, 1999; Papp et al., 2003). Taking all these advantages together, this technology offers many opportunities in fish pathogen diagnosis.

Real-time PCR assays have been developed for accurate detection and/or quantification of specific fish pathogens, including *Aeromonas* spp., *Flavobacterium* spp., *Vibrio* spp., and DNA and RNA viruses (Overturf et al., 2001; Gilad et al., 2004; Balcazar et al., 2007; Kamimura et al., 2007). Examples include a real-time RT-PCR combining reverse transcription for the detection and quantification of the infectious haematopoietic necrosis virus (IHNV), an RNA virus affecting various salmonid species. The assay, using a TaqMan® probe, was 10^3 times more sensitive than the standard RT-PCR (Overturf et al., 2001). Another example includes the development of real-time PCR assays for koi herpes virus (KHV), e.g., the TaqMan® probe-based assay of Gilad et al. (2004). Kamimura et al. (2007) developed a QProbe-based assay for identification and quantification of KHV in fish tissues. Although the sensitivity of this assay was similar to that of a TaqMan® probe PCR, the accuracy of KHV identification and quantification was slightly better with the QProbe in cases of low KHV concentrations (Kamimura et al., 2007).

Loop-mediated isothermal amplification (LAMP). Apart from PCR, other techniques have been developed for the amplification of nucleic acids (Andras et al., 2001). One technique increasingly used in fish pathogen diagnostics includes the loop-mediated isothermal amplification method (LAMP) which rapidly amplifies genomic DNA with high specificity and amplification efficiency under isothermal conditions, avoiding the need of a thermocycler (Notomi et al., 2000). In combination with an additional reverse transcription step, this technique may also be used for RNA viruses (reverse transcription-coupled LAMP; Gunimaladevi et al., 2005; Soliman and El-Matbouli, 2006; Shivappa et al., 2008).

LAMP typically relies on an auto-cycling strand displacement DNA synthesis, per-

formed by a DNA polymerase with high strand displacement activity and a set of four specific primers, as described and well illustrated by Notomi et al. (2000). Although a high specificity is obtained using this method, the selectivity of the technique can be increased by using six primers (Soliman and El-Matbouli, 2005). The entire procedure can be completed in one hour and results in a large amount of stem loop DNAs (Notomi et al., 2000; Savan et al., 2005). Subsequently, these products can be detected by gel electrophoresis resulting in several bands of different sizes for a single sample.

Another method for detection involves real-time detection. As in LAMP, a large amount of DNA is synthesized. Accordingly, a large amount of pyrophosphate ion by-product is generated, yielding an insoluble salt of magnesium pyrophosphate. The presence or absence of target DNA may be judged visually by the appearance of a white precipitate (Caipang et al., 2004) or by turbidity measurement in the reaction mixture (Mori et al., 2001). Alternatively, SYBR® Green may be added to the reaction causing a color change from orange to green in case target DNA is detected (Soliman and El-Matbouli, 2005). The high correlation between turbidity at the end of the reaction and the initial concentration of target DNA makes both qualitative and semi-quantitative diagnosis possible (Caipang et al., 2004).

The detection limit of this technique is similar or better to that of PCR. As a result, LAMP is a rapid, highly specific, sensitive, and cost-effective alternative for PCR which can be used for detection, even on-site detection, of specific fish pathogens (Caipang et al., 2004; Gunimaladevi et al., 2004, 2005; Kono et al., 2004; Soliman and El-Matbouli, 2005, 2006; Yeh et al., 2005, 2006; Sun et al., 2006; Shivappa et al., 2008; Wei et al., 2008). As an example, a LAMP-based method has been developed to rapidly and specifically detect the fish pathogenic bacterium *Edwardsiella ictaluri*, one of the most important pathogens in the aquaculture of channel catfish causing enteric septicemia (Yeh et al., 2005). Likewise, the same authors (Yeh et al., 2006) successfully developed a LAMP assay for the detection of *Flavobacterium columnare*, causative agent of columnaris in many fish species. As an example regarding RNA viruses, Shivappa et al. (2008) developed an RT-coupled LAMP assay that can be used under field conditions for diagnosis of spring viraemia of carp virus (SVCV), a considerable pathogenic agent which causes systemic illness and high mortality in cyprinids, especially in common carp.

Multiplex detection. One limitation of most detection procedures, whether serological or nucleic acid-based, is that only a single or a few targets can be detected and identified in a single assay. As most fish can be infected by a multitude and wide variety of pathogens, with new pathogens being recorded regularly, a comprehensive pathogen screening package would require an endless number of individual tests, making routine screening of multiple targets inefficient, laborious, and expensive. In addition, fish symptoms often result from infection by several pathogens rather than a single pathogen, complicating classical diagnosis. Therefore, multiplex detection enabling detection of numerous pathogens in a single assay has been a major challenge in fish disease diagnostics.

In theory, multiplex detection can be achieved by multiplex PCR (Wilton and Cousins, 1992) using several primer sets in the same reaction targeting discrete pathogens (del Cerro et al., 2002; Gonzalez et al., 2004). However, the development of accurate multiplex formats is often difficult, leads to less sensitive assays, and requires extensive optimization of reaction conditions in order to discriminate at least a few amplicons per reaction. Further, amplicon sizes should be different enough to ensure clear discrimination of the amplicons by gel electrophoresis (Henegariu et al., 1997). This latter limitation does not apply to real-time PCR using specific probes which are labeled with different fluorescent dyes but the limited availability of different fluorophores and the common use of monochromatic light in real-time PCR instruments limit the total amount of PCR reactions that can be performed in a single run (Mackay

et al., 2002). As a result, the maximum number of pathogens detectable in a single assay is currently relatively small using these strategies.

Apart from multiplex PCR, degenerate primers (e.g., for members of a class) have been used, for example, for unambiguous identification of unknown viruses. This strategy is complicated by the existence of highly homologous relatives, making additional procedures such as restriction enzyme analysis, blotting analysis, or cloning and sequencing necessary (Oppegaard and Sorum, 1996; Lilley et al., 1997; Talaat et al., 1997; Heidelberg et al., 2000; Chen et al., 2003).

So far, the most promising technology for the development of multi-pathogen detection systems has been the advent of DNA array technology. In theory, an unlimited number of target organisms can be simultaneously detected and identified using low-density macroarrays (e.g., on a nylon membrane) or high density microarrays (e.g., on a glass slide). In addition, DNA arrays may allow pathogen detection over a wide range of taxonomic levels, even across superkingdom borders (Wilson et al., 2002). Apart from phylogenetic markers, other biomarkers such as virulence genes or antibiotic resistance markers can also be implemented on the array.

Although DNA arrays were originally designed to study gene expression, gene discovery, SNP analysis, and DNA sequencing (Schena et al., 1996; Ramsay, 1998), taxonomists and diagnosticians quickly recognized the potential of this technology for identifying pathogens. With this technology, specific detector oligonucleotides are immobilized on a solid support, essentially allowing reverse dot blot hybridization.

In general, two approaches have been used for signal amplification. The most common involves the use of universal primers that anneal to conserved sequences flanking diagnostic domains in housekeeping genes such as the ribosomal rRNA gene. In this way, numerous targets can be amplified with a single primer pair, while target discrimination is performed afterwards on the array. When targeting different markers, PCR products can be combined at hybridization. In general, short oligonucleotides of approximately 20 nucleotides are used to obtain a high specificity. Indeed, the discriminative power of this approach is very high since even microorganisms whose target sequences differ by a single nucleotide can be discriminated if specific criteria are taken into account (Lievens et al., 2006). This approach is efficient for rapid detection and identification of various microorganisms including bacteria (Call et al., 2003), fungi (Lievens et al., 2003), and some virus groups (Chizhikov et al., 2002). Warsen et al. (2004) developed a DNA microarray based on 16S rDNA sequences for the simultaneous discrimination between 15 economically important fish pathogenic bacterial species among which are several species of *Aeromonas*, *Flavobacterium*, *Mycobacterium*, and *Streptococcus*.

There are many groups of organisms, especially viruses, for which no effective universal primers are available. For these, sequence-nonspecific amplification methods such as strategies based on the random primed amplification method of Bohlander et al. (1992) may be used in combination with arrays of 70-mer oligonucleotides to identify sequences of numerous unrelated targets (Wang et al., 2002; Agindotan and Perry, 2007). The most remarkable application of this technology has been the development of a microarray for the detection and genotyping of over 1000 viruses, including those from fish (Wang et al., 2003).

Major advantages of the first approach over the second one are its higher sensitivity, which is comparable to the sensitivity of other molecular techniques (Lievens et al., 2003), and its higher specificity (Levesque et al., 1998). The advantage of the second approach is that non-related sequences can be simultaneously amplified in a relatively simple and cost-effective way.

Another strategy of multiplexing with high levels of specificity (and sensitivity) is the use of multiplex PCR primers that amplify discrete targets followed by amplicon discrimination using DNA arrays (Gonzalez et al., 2004). Li et

al. (2001) showed that this detection technique is also possible for discriminating RNA viruses and, in particular, to type multiple strains of the influenza virus. Nevertheless, the development of an efficient reaction in which all targets are amplified with the same efficiency is not always straightforward and requires extensive optimization of the reaction conditions.

Discussion and Future Directions

Laboratories that provide diagnostic services and inspection agencies are increasingly searching for fast routine methods that provide rapid detection and reliable identification of pathogenic organisms, including fish pathogens. In this regard, PCR-based methods such as (real-time) PCR are more and more implemented in practice. Nevertheless, to increase efficiency and reduce costs, time, and labor, multiplex detection assays are desirable. Currently, DNA array technology is the most suitable technique to simultaneously detect numerous targets. This technology can also be used for pathogen quantification since hybridization signals are proportional to the quantity of target DNA (Lievens et al., 2005b), making this technique even more attractive for fish pathogen diagnosis and disease management decisions. However, as the amount of material necessary for analysis becomes less with the development of more sensitive technologies such as those based on PCR, development of appropriate sampling strategies and knowledge of the disease development will become more challenging.

So far, molecular diagnostics are relatively expensive in terms of investment and facilities. Consequently, they are pertinent only for well-equipped laboratories. The next challenge is to simplify molecular diagnostics and bring them into the field, enabling on-site pathogen diagnosis. Antibody-based lateral flow devices, originally developed for pregnancy testing, can meet these demands (Smits et al., 2001) and are increasingly being developed for on-site diagnosis of fish-related diseases. A recent example includes a lateral flow assay for rapid detection of the infectious salmon anaemia virus, ISAV (Aquatic Diagnostics Ltd., Stirling, UK). A disadvantage of such assays, however, is relatively low sensitivity, limiting its widespread use. Nevertheless, since these tests are relatively inexpensive, take little time to perform, and do not require specialized equipment or knowledge, there is growing interest in the use of these tests for on-site, front-line pathogen screening.

Nucleic acid-based detection platforms are also becoming available for on-site pathogen diagnosis. One example includes the development of portable real-time PCR instruments such as the SmartCycler (Cepheid, Sunnyvale, CA, USA), which enables parallel testing of 16 samples under different conditions.

These new developments pose new challenges for sample processing as several limitations inherent to field-testing need to be circumvented. For example, PCR reagents need to be stable at ambient temperature (Tomlinson et al., 2005).

Regarding multiplex technologies, progress can be expected from PCR arrays that combine the advantages of DNA arrays and real-time PCR, resulting in high throughput capacity and accurate quantification (Belgrader et al., 1998). Typically, PCR arrays provide a platform on which spatially separated PCR reactions can be performed simultaneously. This can be exemplified by the OpenArray™ technology of BioTrove (Woburn, MA, USA) in which a few thousand real-time PCR assays (48 × 64 reactions) can be performed at the same time in minuscule reaction holes. However, the sensitivity and accuracy of quantification may suffer from the ultralow reaction volumes used. Another interesting development is the lab-on-chip instrument (Anderson et al., 2000; Wang, 2000), which combines several handlings (from DNA extraction to DNA analysis) on a single, portable, and fully automated device.

Which technologies will eventually be implemented in fish pathogen diagnosis remains unclear, but obviously only those assays that become available at an affordable price. Taking into account the unlimited expansion possibilities of DNA arrays to include oligonucleotides for all markers of

interest and current technical and economic requirements for routine diagnostics, we believe that as soon as DNA array-based detection procedures become more automated, DNA macroarrays may become the new benchmark in fish pathogen diagnosis. Compared to DNA microarrays, macroarrays are cheaper, more sensitive, and can be reused many times (Lievens and Thomma, 2005), favoring the use of this type of DNA arrays.

Even in its current format, DNA macroarrays are routinely used by several diagnostic laboratories. For example, laboratories are increasingly using routine DNA arrays for plant pathogen detection, using for example the DNA Multiscan®, a membrane-based DNA array to detect and identify over 75 plant pathogenic fungi, oomycetes, and bacteria (www.DNAmultiscan.com; Lievens and Thomma, 2005).

Likewise, we are now developing a DNA macroarray for a comprehensive set of economically important fish pathogens for the ornamental fish industry. In Fig. 1, a first generation DNA array containing 16S rDNA oligonucleotides for the identification of 15 fish pathogenic bacterial species is shown. The new diagnostic assay will eventually contain detector oligonucleotides for a diverse set of fish pathogens, including multiple species from *Aeromonas*, *Edwardsiella*, *Flavobacterium*, *Mycobacterium*, *Pseudomonas*, *Renibacterium*, *Vibrio*, and *Yersinia,* and a selection of viral pathogens, e.g., koi herpes virus (KHV), carp pox virus (CPV), channel catfish virus (CCV), white spot syndrome virus (WSSV), spring viraemia of carp virus (SVCV), viral haemor-

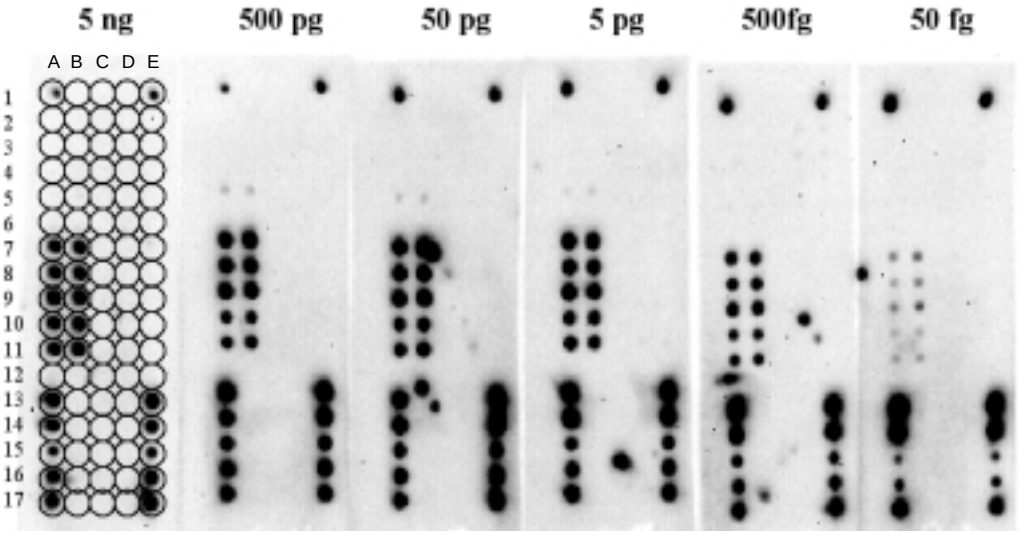

Fig. 1. Identification of a fish pathogenic bacterium (*Pseudomonas anguilliseptica,* the causative agent of pseudomonadiasis) using a 16S rDNA sequence-based DNA macroarray. Each detector oligonucleotide is spotted in duplicate on a nylon membrane. Specificity of the analysis is enhanced by using multiple oligonucleotides for each target species. In addition to the immobilized target-specific sequences, the array contains control oligonucleotides for hybridization (13A, 14A, 15A, 16A, 13E, 14E, 15E, and 16E) and a reference for detection and calibration (1A, 1E, 17A, and 17E). PCR-labeled amplicons generated with universal primers hybridize to species-specific oligonucleotides for *P. anguillispetica* (7A, 8A, 9A, 10A, 11A, 7B, 8B, 9B, 10B, and 11B). Based on the location of the signals, identification is performed. Results are shown for different amounts of genomic DNA that have been amplified (ranging 5 ng to 50 fg) and hybridized to the array.

rhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), and infectious salmon anaemia virus (ISAV). As soon as other interesting biomarkers such as virulence genes or antibiotic resistance markers become available, they will be implemented in the assay. Ultimately, this array could be used for rapid comprehensive fish pathogen diagnosis and preventing diseases as well as in biosafety and biosecurity programs.

Acknowledgements

The authors would like to thank the 'Vlaams Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen' (IWT-070113) and the industrial sponsors for their financial support.

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