Molecular Diagnosis of Fish Diseases: a Review

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Abstract

Molecular techniques are potentially faster and more sensitive than culture, serology, and histology methods that are traditionally used to identify fish pathogens. During the last 15 years or so, molecular techniques have been increasingly employed to diagnose fish diseases. These techniques include polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization, in situ hybridization, and microarray. Pathogens can be detected from asymptomatic fish by molecular diagnostic techniques so disease outbreak could be prevented. Thus antibiotic treatment can be reduced so that creation of antibiotic resistant bacteria may be eliminated. In this paper molecular techniques for detection of fish pathogens are reviewed and the potential for their application are discussed. The application of new techniques as a routine tool in a diagnostic laboratory is an area where relevant literature is scarce and this may contribute to the reticence of some to adopt these methods.

Key Words: Molecular techniques, fish diseases, antibiotic resistance, molecular diagnosis.

1. Introduction

Under ideal environmental conditions, healthy looking fish without a clinical sign or lesion can carry pathogens that create serious risks for the spread of contagious diseases in the fish populations. Disease becomes evident only when stressful condition occurs. Under intensive aquaculture conditions the risk of stress increases and a significant proportion of the stock may become infected. Therefore, detection of pathogen from carrier fish is essential for the effective fish disease control. Since most of the time, prevalence of diseases may change depending on time of the year and water temperature (Plumb, 1999), it may be difficult to sample an infected fish from a population. Statistically, relevant disease surveillance and monitoring requires testing large numbers of fish. Reliable detection of fish pathogens in a fish population is difficult if fish with disease are not available or only a low percentage of the fish is infected. The probability of detecting pathogen from clinically normal fish is increased by testing larger numbers of fish; the number of fish that should be tested depends on the level of probability desired, the population size, and the assumed prevalence of infection. For example, a random sample of 145 fish is required to obtain a probability of detecting at least one infected fish from a population of 2000 fish if 2% of the population is infected (AFS-FHS, 2003). Therefore, to detect pathogen carrier fish, a cost-effective, so sensitive, and specific system is required for surveillance and monitoring fish population.

Traditionally the diagnosis of the disease is carried out by agar cultivation and then phenotypic and serological properties of the pathogen or histological examination (Bernardet *et al*., 1990; Pazos *et al*., 1996). Furthermore, some of the bacteria could not be differentiated by conventional diagnostic methods from other phenotypically similar bacteria of the same genera (Shewan and McMeekin, 1983). Some attempts have been made using biochemical tests, DNA homology and protease variability (Pyle and Shotts, 1980; Bertolini and Rohovec, 1992; Chen *et al*., 1995), but these techniques have some disadvantages such as need for previous isolation of the pathogen and insufficient sensitivity to detect low levels of pathogen.

Last fifteen years or so, great advances have taken place in understanding the molecular biology of fish pathogens and their hosts, and molecular biology has become a routine tool in the search for improved methods of diagnosis and control of fish diseases and the epidemiology of bacterial, viral, and parasitical diseases. Detection of nucleic acid molecules has demonstrated its usefulness for highlighting hardly cultivable, non-cultivable, and even dead microorganisms, generating appropriate novel or replacement technologies.

Molecular techniques can be used to solve that type of problems and increase sensitivity and specificity of pathogen detection. These techniques include polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization, in situ hybridization, and microarray. Since molecular diagnostic techniques are faster and more sensitive

than conventional diagnostic techniques, pathogens can be detected from asymptomatic fish, so disease outbreak could be prevented. Thus antibiotic treatment can be reduced therefore; creation of antibiotic resistant bacteria may be eliminated.

Antimicrobial resistance of bacteria in the fish farms has been intensively studied (Alderman and Hastings, 1998). In the aquaculture, antimicrobial agents are released into the surrounding water during medical treatment of bacterial fish diseases (Bjørklund *et al*., 1990). A high incidence of bacteria resistant to the antimicrobials used in aquaculture have been found in fish farms and the surrounding aquatic environments (McPhearson *et al*., 1991; Sandaa *et al*., 1992; DePaola *et al*., 1995; Schmidt *et al*., 2000), and also antimicrobials residues have been detected in the sediments of marine fish farms (Björklund *et al*., 1990). Overfeeding and water currents around marine fish farms stimulate antimicrobials build up in the sediment (Coyne *et al*., 1994). Numerous investigators have attempted to elucidate the occurrence and persistence of antibiotic resistance, mostly in marine aquaculture production systems (Herwig *et al*., 1997).

Antibiotic residues may accumulate in integrated fish farms when the ponds are only or rarely emptied at the time of fish harvest. That kind of build up could create selective pressure on the bacteria by favoring selection and growth of antibiotic-resistant bacterial strains. Although increased levels of antibiotic resistance in and around the fish farms may only occur rapidly, there is a potential risk that antibiotic resistance genes could be spread into a wide range of aquatic bacteria (Petersen *et al*., 2002). The use of antibiotics as growth promoters or therapeutic agents in animal husbandry has been linked to certain antimicrobial resistance patterns among human bacterial pathogens (Bager *et al*., 1997; Wegener *et al*., 1999), suggesting that there is a possible flow of antimicrobial resistance genes between animal and human pathogens. Potential transfer of resistant bacteria and resistance genes from aquaculture environments to humans may occur through direct consumption of antimicrobial-resistant bacteria present in fish and associated products. The apparent increase of the occurrence of antibiotic resistance among bacteria from various areas of animal production during the past years and its possible implications for public health have in many countries lead to an intensified surveillance of bacterial resistance. The relationship between antibiotic usage and resistance is strongly supported by data from several studies which show the presence of an important population of antibiotic resistant bacteria in the microflora of fish farms (Kruse and Sorum, 1994; Adams *et al*., 1998; Alderman and Hastings, 1998). Since molecular diagnostic techniques are faster and more sensitive than conventional diagnostic techniques, pathogens can be detected from asymptomatic fish so disease outbreak could be prevented. The aims of this review are: 1) to evaluate molecular techniques for the detection and identification of bacterial and viral fish pathogen, 2) to explain how to eliminate contaminations among samples during necropsy and PCR.

2. Molecular tools for fish diseases diagnosis

Disease management and assessment of cultured fish is a major concern to commercial aquaculturists. The ability to identification of the presence or absence of a pathogenic organism in fish quickly would have significant economic benefits. An additional benefit would be if the concentration of the infectious organism could be determined in the fish or the environment so that changes in abundance of these organisms could be monitored. Finally, the development of a system that could accurately assess the carrier state of fish within an area containing a disease causative agent would aid in the development of management programs.

Great advances have been made in improving the sensitivity and specificity of diagnosis of bacterial, viral, and parasitical fish diseases. In the molecular techniques, typically, DNA is extracted from the sample of interest which can be probed by DNA hybridization and analyzed by restriction fragment length polymorphism (RFLP). More commonly, DNA is amplified by the polymerase chain reaction (PCR) using specific primers for diagnostic sequences. This may be followed by RFLP, PCR linked to hybridization with specific oligoprobes or, non-specific primers used to produce random amplified polymorphic DNA (RAPD) (Prichard, 1997; McKeand, 1998).

2.1. Polymerase chain reaction

Polymerase chain reaction is a technique for amplifying a specific region of DNA, defined by a set of two "primers" at which DNA synthesis is initiated by a thermostable DNA polymerase. Usually, at least a million-fold increase of a specific section of a DNA molecule can be realized and the PCR product can be detected by gel electrophoresis. The regions amplified are usually between 150-3,000 base pairs (bp) in length. (McPherson *et al*., 1991). Primer design is important to obtain greatest possible sensitivity and specificity. Therefore, the primers should be sufficiently long to allow a high annealing temperature and reduce the opportunity for nonspecific primer annealing, but primers that are too long may facilitate nonspecific annealing even to regions of DNA that are not perfectly complementary to the primer sequence. The reaction includes template DNA that may be in various forms, from a simple tissue lysate to purified DNA, primers, polymerase enzyme to catalyze creation of new copies of DNA, and nucleotides to form the new copies. During each round of the thermocycling reaction, the

template DNA is denatured, primers anneal to their complementary regions and polymerase enzyme catalyses the addition of nucleotides to the end of each primer, thus creating new copies of the target region in each round. Theoretically, the increase in amount of product after each round will be geometric.

Reverse transcriptase polymerase chain reaction (RT-PCR) is used to detect specific mRNA and determine levels of gene expression (Koo and Jaykus, 2000). Compared to the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and RNase protection assay, RT-PCR can be used to quantify mRNA levels from much smaller samples. In fact, this technique is sensitive enough to enable quantitation of RNA from a single cell. Over the last several years, the development of novel chemistries and instrumentation platforms enabling detection of PCR products on a real-time basis has led to widespread adoption of real-time RT-PCR as the method of choice for quantitative changes in gene expression. Furthermore, real-time RT-PCR has become preferred method for validating results obtained from array analyses and other techniques that evaluate gene expression changes on a global scale. The sensitivity and specificity achieved in a well-designed RT-PCR make it an ideal tool for use in the surveillance and monitoring of covert infections.

As in the eukaryotes, the prokaryotic rRNA genes contain highly conserved sequences. The potential utility of conserved regions to identify or amplify the rRNA genes, followed by exploitation of more variable regions of the genes or spacers to detect or identify bacteria that may be difficult or even impossible to culture has long been recognized (Barry *et al*., 1990). The rRNA genes have been used in PCR assays for *Renibacterium salmoninarum* (Rhodes *et al*., 1998), *Aeromonas salmonicida* (Høie *et al*., 1999) and *Yersinia ruckeri* (Altinok *et al*., 2000). The same methods are employed in the detection and study of significant pathogens that are not included in European Community legislation. These include *Vibrio anguillarum* (Pedersen *et al*., 1999), *Lactococcus garvieae* (Zlotkin *et al*., 1998), *Piscirickettsia salmonis* (Marshall *et al*., 1998), *Flexibacter*, (Bader and Shotts, 1998), *Flavobacterium*, (Izumi and Wakabayashi, 2000) *Photobacterium* (Osorio *et al.,* 1999) and *Mycobacterium* (Patel *et al*., 1997).

2.2. Multiplex PCR

New developments such as design of PCR conditions that can detect several pathogens at one time in a multiplex reaction will improve time and cost-efficiency of this methodology, countering one of the major arguments against the adoption of these techniques as routine (Williams *et al*., 1999). In multiplex PCR more than one target sequence can be amplified by including more than one pair of primers in the reaction. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility. Since its introduction, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics, including gene deletion analysis (Chamberlain *et al*., 1988; Chamberlain *et al*., 1989), quantitative analysis (Zimmermann *et al*., 1996; Rithidech *et al*., 1997), and RNA detection (Zou, 1997). In the field of infectious diseases, the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi and parasites.

2.3. Labeling and detection of nucleic acids

A variety of labeling and detection systems exist for nucleic acid probes (Tijssen, 1993). Radioisotopes were once the norm but, in the interests of researcher safety, other methods are becoming increasingly popular. The variety of labels and detection methods now available can provide a system suitable for any application, from dot blots to in situ hybridization. These include labeling with a variety of haptens such as biotin or digoxygenin and detection by antibody binding coupled with fluorescent, chemiluminescent or colorimetric detection methods (Deering *et al*., 1991; Hariharan *et al*., 1995; Tyagi and Kramer, 1996; Gonzalez *et al*., 1997).

2.4. Restriction enzyme digestion

Restriction enzymes (or restriction endonucleases) cleave DNA in a very specific fashion. Type II restriction enzymes, most commonly used for DNA analysis and genetic engineering, each have a unique nucleotide sequence at which it cuts a DNA molecule. A particular restriction enzyme will cleave DNA at that only recognition sequence that is often a six base pair palindromic sequence, but others recognize four or even eight base pair sequences. A common use for restriction enzymes is to generate a "fingerprint" of a particular DNA molecule. Because of the sequence specificity of restriction enzymes, these enzymes can cut DNA into discrete fragments which can be resolved by gel electrophoresis. This pattern of DNA fragments generates a "DNA fingerprint" and each DNA molecule has its own fingerprint. Other restriction enzymes can be used to further characterization of a particular DNA molecule. The location of these restriction enzyme cleavage sites on the DNA molecule can be compiled to create a restriction enzyme map (Grizzle *et al.*, 2002). These maps are very useful for identifying and characterizing a particular DNA plasmid or region.

Restriction enzymes recognize specific short sequences of DNA and cleave the DNA at that site. Single nucleotide changes can result in the gain or loss of a restriction site, thus altering the number of fragments produced following digestion of DNA. These RFLP can be visualized following gel electrophoresis of the digested DNA to separate the

fragments according to size. Differences in the RFLP profiles have revolutionized criminal investigations and have become powerful tools in the identification of individuals in paternity and maternity cases, population genetics, and in the diagnosis of a variety of diseases.

2.4.1. Restriction Fragment Length Polymorphism (RFLP)

RFLP is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms 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.

Isolation of sufficient DNA for RFLP analysis is time-consuming and labor intensive. However, PCR can be used to amplify very small amounts of DNA, usually in 2-3 hours, to the levels required for RFLP analysis. Therefore, more samples can be analyzed in a shorter time.

2.4.2. Amplified Fragment Length Polymorphism (AFLP)

A rapid PCR-based technique, AFLP can be used for typing prokaryotes and eukaryotes. The method is based on the selective PCR amplification of genomic restriction fragments of the whole genome (Vos *et al*., 1995) and has been shown to be rapid, reproducible, and highly discriminatory (Boumedine and Rodolakis, 1998). Selected markers are amplified in a PCR, which makes AFLP an easy and fast tool for strain identification in agriculture, botany, microbiology, and animal breeding. The AFLP method used was essentially that described by Valsangiacomo *et al*., (1995).

AFLP analysis belongs to the category of selective restriction fragment amplification techniques, which are based on the ligation of adapters to genomic restriction fragments followed by a PCR-based amplification with adapter-specific primers (Vaneechoutte, 1996). For AFLP analysis, only a small amount of purified genomic DNA is needed; this is digested with two restriction enzymes, one with an average cutting frequency (like *Eco*RI) and a second one with a higher cutting frequency (like *Mse*I or *Taq*I). Double-stranded oligonucleotide adapters are designed in such a way that the initial restriction site is not restored after ligation, which allows simultaneous restriction and ligation, while religated fragments are cleaved again. An aliquot is then subjected to two subsequent PCR amplifications under highly stringent conditions with adapter-specific primers that have at their 3' ends an extension of one to three nucleotides running into the unknown chromosomal restriction fragment (Ajmone-Marsan *et al.,* 1997).

Alternative AFLP typing procedures are based on one enzyme with a single adapter and analysis by agarose gel electrophoresis (Gibson *et al.,* 1998). A major improvement has been obtained by switching from radioactive to fluorescently labeled primers for detection of fragments in an automatic sequence apparatus (Koeleman *et al*., 1998). In addition, it has been shown that for small bacterial and fungal genomes a single PCR amplification with one and two selective nucleotides, respectively, on both primers are sufficient (Mueller *et al*., 1996; Koeleman *et al*., 1998; Duim *et al*., 1999).

2.4.3. Random Amplified Polymorphic DNA (RAPD)

The technically demanding method of RAPD has been applied to the study of crayfish plague fungus, *Astacus astaci* (Huang *et al.,* 1994). RAPD uses a single primer in low-stringency polymerase chain reactions (Welsh and McClelland, 1990). Random binding of primers results in different sizes of fragments from samples with nonidentical DNA. Application of the RAPD technique grouped different isolates of the fungus and provides the means to carry out epidemiological investigations (Lilley *et al*., 1997; Oidtmann *et al*., 1999). The method has also been used to examine another *Aphanomyces* species that has resulted in serious losses in both farmed and wild fish in Asia (Lilley *et al*., 1997). Other fish pathogens have been studied using RAPD, but problems with reproducibility and risks of contamination render the method unsuitable as a stand-alone method of diagnosis. However, RAPD can be a useful technique as a first step in the development of specific primers or probes and has been used in such a way in the study of bacteria.

2.5. In Situ Hybridization

In situ PCR has become a powerful molecular tool in research as well as clinical practice. This technique has resulted in an increased understanding of infectious and neoplastic diseases and improvements in diagnosis of disease. In situ RT-PCR gives more detailed information by allowing for highly sensitive detection of low abundance gene expression in a given cell while providing anatomical information. The usefulness of these techniques has been hampered by low detection sensitivity, poor reproducibility, and high backgrounds. Moreover, many of the methods used to visualize the results of PCR amplification within cells and tissues employ radioactive tracers, making performance of the techniques cumbersome and costly.

Researchers have developed a method for specific fluorescent detection of gene expression using in situ RT-PCR. This method enables the

researcher or clinician to detect low levels of gene expression within tissues with very low background interference while addressing many of the other existing drawbacks to using in situ RT-PCR. Potential Areas of Application are detection and diagnosis of viruses and other infectious agents in specific cell types within tissues, detection, and characterization of tumor cells within a tissue, detection, and diagnosis of genetic mutations in inherited diseases, and detection of genes and gene expression in tissue.

Fluorescence *in situ* hybridization, or FISH, is a method used to label cells or chromosomes according to the sequences of nucleic acids contained within them. In microbiology, the nucleic acid that is labelled as RNA or DNA of the ribosomes and the target is usually whole cells. The process works by taking fluorescently labelled pieces of DNA or RNA called probes that are around 20 nucleotides in length. The probes are incubated in the presence of cells under appropriate conditions to permit specific hybridization of probe to target nucleic acid. Cells types that contain ribosomes with complementary RNA sequences become labeled by the binding of the fluorescent probe in situ. These labelled cells can then be visualized by flow cytometric or fluorescence microscopy.

2.6. DNA microarrays

There are a number of ways of using DNA microarrays for the detection of unique DNA (or RNA) sequences. One method is to fluorescently label all the DNA sequences in the test sample. The sample DNA that hybridizes to a specific location on the microarray can be detected by fluorescent array detection and the data analyzed by computer programs. Often more practical is to use competitive hybridization in which the test sample competes for hybridization to the tethered oligonucleotide, on the chip, with a fluorescent labeled competitor oligonucleotide. When the test DNA is perfectly complimentary to the tethered oligonucleotide, it will hybridize to the chip. When the test DNA is not perfectly complementary to the tethered oligonucleotide, the fluorescent labeled competitor oligonucleotide will bind to the tethered oligonucleotide on the chip and displace the test DNA. A fluorescent microarray detector and computer program can then analyze the fluorescent array for the presence or absence of the species/strain specific DNA sequence.

Compared with traditional nucleic acid hybridization with membranes, microarrays offer the additional advantages of high density, high sensitivity, rapid detection, lower cost, automation, and low background levels (Shalon *et al*., 1996). Microarrays may provide a better option for large-scale diagnostic testing and can survey a sample for a multitude of sequences simultaneously (Southern, 1996; Mir, 2000). Since most of the pathogens genetic sequences are available in the GenBank, oligonucleotide probes complementary to all pathogens can be made and inserted into microarray so that variety of microbes could be detected by a single microarray chip. As a result, microarray-based technology is potentially well suited for identifying fish pathogens in fish populations. The microarray techniques does not require such sequence conservation, however, because all of the diverse gene sequences from different populations of the same functional group can be fabricated on arrays and used as probes to monitor contagious fish disease especially during the asymptomatic period of the diseases (Zhou and Dorothea, 2004). Microarrays are already proving valuable for assaying gene expression. A large number of probes on an array can reveal which genes are expressed or are present in the sample. This type of array would be particularly useful in studies of pathogens, where the presence of certain genes or gene products indicate whether the organism is pathogenic or not. Set up cost for the use of DNA microarrays is high. However, once the equipment is available and microarrays have been prepared, cost per unit of sample analyzed will be low. Furthermore, analysis time is extremely short. DNA microarray technology will be used in the future for fish diseases diagnosis especially during the asymptomatic period of diseases.

3. Potential problems and avoiding contamination

The choice of tissue sampled may have a significant effect on PCR results. Various substances such as hemoglobin, bacterial constituents, and high concentrations of no target DNA can inhibit amplification (Wilson, 1997). High concentrations of DNA from the host and high numbers of fish cells can inhibit amplification of *A. salmonicida* DNA (Høie *et al.,* 1997) and are also likely to inhibit other amplifications. The impact of PCR inhibitors such as selective components and fish tissues can be minimized by using a high-throughput and high-quality nucleic acid extraction system.

To eliminate contamination during necropsy and PCR, use new (disposable) dissecting tools for each fish or clean dissecting equipment between fish should be used. Equipment disinfection should include both manual cleaning of equipment to remove all tissue from crevices of dissecting tools and use of a chemical agent such as bleach followed by a series of rinses in water. Alcohol and flaming are not satisfactory for this method because they do not reliably destroy DNA. If samples need to be homogenized, to avoid cross contamination between samples use stomacher instead of polytron because alcohol treatment of polytron will not destroy the bacteria and virus especially enveloped virus. To avoid cross contamination between samples, a different room should be used for each of the

following steps: (1) necropsy (2) DNA extraction, (3) PCR master mix preparation, (4) DNA quantification and addition of DNA to PCR mixture, and (5) thermocycler and electrophoresis. Laboratory coats, gloves, and pipettes should not be taken from one room to another. Uracil-DNA glycosidase (UNG) should be used with dUTP to eliminate PCR contamination with the reaction product. UNG degrades uracil-containing DNA at 20°C and is inactivated at elevated temperatures. To check the efficiency and the sensitivity of the PCR, it is advisable to apply standard molecules as indicators of the procedures. It is important to detect false negative results depending on pipetting errors or inhibition of the amplification, often observed when PCR is performed with complex samples, such as blood and tissues. To eliminate false negative PCR results internal control such as β-actin gene primers should be used to amplify β-actin gene. The size difference allowed easy discrimination between their PCR products. Using the internal control undoubtedly facilitated the interpretation of negative PCR results and it was easy to identify samples which were inhibiting the amplification (Grizzle *et al*., 2003).

4. Conclusions

Molecular tools are increasingly relevant to fish diseases. The sequencing of the complete genomes of pathogens is allowing great advances in studying the biology, and improving diagnosis and control of pathogens. Using nucleic acid as targets, and new methods of analyzing polymorphism in this nucleic acid, can improve specificity, sensitivity, and speed of diagnosis and offer means of examining the relationships between genotype and phenotype of various pathogens. Progress in techniques aids epidemiological studies as well as identifying causes of disease outbreaks or the presence of pathogens. Therefore, molecular biology can be a routine tool in the search for improved methods of diagnosis and control of fish pathogens and the epidemiology of infectious fish diseases. However, in Turkey the applications of these techniques on a routine basis in diagnostic laboratories are few. The time has now come for their application in the diagnosis of diseases in aquaculture.

5. References

Adams, C.A., Austin, B., Meaden, P.G. and McIntosh, D. 1998. Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. App. Environ. Microbiol., 64: 4194-4201.

AFS-FHS (American Fisheries Society – Fish Health Section). 2003. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 5th edition. Fish Health Section, American Fisheries Society, Bethesda, Maryland, USA.

Ajmone-Marsan, P., Valentini, A., Cassandro, M.G., Vecchiotti-Antaldi, Bertoni, G. and Kuiper, M. 1997. AFLP markers for DNA fingerprinting in cattle. Anim. Genet., 28: 418-426.

Alderman, D.J. and Hastings, T.S. 1998. Antibiotic use in aquaculture: development of antibiotic resistance-potential for consumer health risks., Int. J. Food Sci. Tech., 33: 139–155.

Altinok, I., Grizzle, J.M. and Liu, Z. 2000. Detection of *Yersinia ruckeri* in rainbow trout blood by use of polymerase chain reaction. Dis. Aquat. Org., 44: 29-34.

Bader, J.A. and Shotts, E.B. 1998. Determination of phylogenetic relationships of *Flavobacterium psychrophilum* (*Flexibacter psychrophilus*), *Flavobacterium columnare* (*Flexibacter columnaris*) and *Flexibacter maritimus* by sequence analysis of 16S ribosomal RNA genes amplified by polymerase chain reaction. J. Aquat. Anim. Health, 10: 320–327.

Bager, F., Madsen, M., Christensen, J. and Aarestrup, F.M. 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* in Danish poultry and pig farms. Preven. Vet. Med., 31: 95-112.

Barry, T., Powell, R. and Gannon, F. 1990. A general method to generate DNA probes for microorganisms. Bio/Tech., 8: 233–236.

Bernardet, J.F., Campbell, A.C. and Buswell, J.A. 1990. *Flexibacter maritimus* is the agent of 'black patch necrosis' in Dover sole in Scotland. Dis. Aquat. Org., 8: 233-237.

Bertolini, J.M. and Rohovec, J.S. 1992. Electrophoretic detection of proteases from different *Flavobacterium columnare* strains and assessment of their variability. Dis. Aquat. Org., 12: 121-128.

Björklund, H., Bondestam, J. and Bylund, G. 1990. Residues of oxytetracycline in wild fish and sediments from fish farms. Aquaculture, 86: 359-367.

Boumedine, K.S. and Rodolakis, A. 1998. AFLP allows the identification of genomic markers of ruminant *Chlamydia psittaci* strains useful for typing and epidemiological studies. Res. Microbiol., 149: 735-744.

Chamberlain, J.S., Gibbs, R.A., Ranier, J.E., Nguyen, P.N. and Caskey, C.T. 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res., 16: 11141-11156.

Chamberlain, J.S., Gibbs, R.A., Ranier, J.E., Nguyen, P.N. and Caskey, C.T. 1989. Multiplex PCR for the diagnosis of Duchenne muscular dystrophy, D.H. Gelfand, M.A. Innis, J.J. Shinsky, and T.J. White (Eds.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, California: 272-281

Chen, M.F., Henry-Ford, D. and Groff, J.M. 1995. Isolation and characterization of *Flexibacter maritimus* from marine fishes of California. J. Aquat. Anim. Health, 7: 318-326.

Coyne, R., Hiney, M., O'Connor, B., Kerry, J., Cazabon, D. and Smith, P. 1994. Concentration and persistence of oxytetracycline in sediments under a marine salmon farm. Aquaculture, 123: 31-42.

Deering, R.E., Arakawa, C.K., Oshima, K.H., O'Hara, P.J., Landolt, M.L. and Winton, J.R. 1991. Development of a biotinylated DNA probe for detection and

identification of infectious hematopoietic necrosis virus. Dis. Aquat. Org., 11: 57–65.

DePaola, A., Peeler, J.T. and Rodrick, G.E. 1995. Effect of oxytetracycline-medicated feed on antibiotic resistance of gram-negative bacteria in catfish ponds. App. Env. Microbiol., 61: 2335-2340.

Duim, B., Wassenaar, T.M., Rigter, A. and Wagenaar, J.A. 1999. High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with AFLP fingerprinting. Appl. Environ. Microbiol., 65: 2369-2375.

Gibson, J.R., Slater, E., Xerry, J., Tompkins, D.S. and Owen, R.J. 1998. Use of an amplified-fragment length polymorphism technique to fingerprint and differentiate isolates of *Helicobacter pylori*. J. Clin. Microbiol., 36: 2580-2585.

Gonzalez, M.P., Sanchez, X., Ganga, M.A., Lopez-Lastra, M., Jashes, M. and Sandino, A.M. 1997. Detection of the infectious hematopoietic necrosis virus directly from infected fish tissues by dot blot hybridisation with a non-radioactive probe. J. Virol. Methods, 65: 273–279.

Grizzle, J.M., Altinok, I., Fraser, W.A. and Francis-Floyd, R. 2002. First isolation of largemouth bass virus. Dis. Aquat. Org., 50: 233-235.

Grizzle, J.M., Altinok, I. and Noyes, A.D. 2003. PCR method for detection of largemouth bass virus. Dis. Aquat. Org., 54: 29-33.

Hariharan, H., Qian, B., Despres, B., Kibenge, F.S., Heaney, S.B. and Rainnie, D.J. 1995. Development of a specific biotinylated DNA probe for the detection of *Renibacterium salmoninarum*. Can. J. Vet. Res., 59: 306–310.

Herwig, R.P., Gray, J.P. and Weston, D.P. 1997. Antibacterial resistant bacteria in surficial sediments near salmon net-cage farms in Puget Sound, Washington. Aquaculture, 149: 263-283.

Høie, S., Dalsgaard, I., Aase, I.L., Heum, M., Thornton, J.M. and Powell, R. 1999. Polymerase chain reaction (PCR)-based typing analysis of atypical isolates of the fish pathogen *Aeromonas salmonicida*. Syst. Appl. Microbiol., 22: 403–411.

Høie, S., Heum, M. and Thoresen, O.F. 1997. Evaluation of a polymerase chain reaction-based assay for the detection of *Aeromonas salmonicida salmonicida* in Atlantic salmon *Salmo salar*. Dis. Aquat. Org., 30: 27–35.

Huang, T.S., Cerenius, L. and Soderhall, K. 1994. Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. Aquaculture, 126: 1–10.

Izumi, S. and Wakabayashi, H. 2000. Sequencing of gyrB and their application in the identification of *Flavobacterium psychrophilum* by PCR. Fish Pathol., 35: 93–94.

Koeleman, J.G.M., Stoof, J., Biesmans, D.J., Savelkoul, P.H.M. and Vandenbroucke-Grauls, C.M.J.E. 1998. Comparison of ARDRA, RAPD and AFLP fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii*. J. Clin. Microbiol., 36: 2522-2529.

Koo, K. and Jaykus, L.-A. 2000. Selective amplification of bacterial RNA: use of a DNA primer containing mismatched bases near its 3' terminus to reduce false-positive signals. Lettters App. Microbiol., 31: 187-192.

Kruse, H. and Sorum, H. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. App. Environ. Microbiol., 60:4015-4021.

Lilley, J.H., Cerenius, L. and Soderhall, K. 1997. RAPD evidence for the origin of crayfish plague outbreaks in Britain. Aquaculture, 157: 181-185.

Marshall, S., Heath, S., Henriquez, V. and Orrego, C. 1998. Minimally invasive detection of *Piscirickettsia salmonis* in cultivated salmonids via PCR. Appl. Environ. Microbiol., 64: 3066–3069.

McKeand, J.B. 1998. Molecular diagnosis of parasitic nematodes. Parasitology, 117: 87–96.

McPhearson, R.M., DePaola, A., Zywno, S.R., Motes, M.L., Jr. and Guarino, A.M. 1991. Antibiotic resistance in Gram-negative bacteria from cultured catfish and aquaculture ponds. Aquaculture, 99: 203-211.

Mir, K.U. 2000. The hypothesis is there is no hypothesis. Trends Genet., 16(2): 63–64.

Mueller, U.G., Lipari, S.E. and Milgroom, M.G. 1996. Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*. Mol. Ecol., 5: 119-122.

Oidtmann, B., Cerenius, L., Schmid, I., Hoffmann, R. and Soderhall, K. 1999. Crayfish plague epizootics in Germany-classification of two German isolates of the crayfish plague fungus *Aphanomyces astaci* by random amplification of polymorphic DNA. Dis. Aquat. Org., 35: 235–238.

Osorio, C.R., Collins, M.D., Toranzo, A.E., Barja, J.L. and Romalde, J.L. 1999. 16S rRNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. Appl. Environ. Microbiol., 65: 2942–2946.

Patel, S., Yates, M. and Saunders, N.A. 1997. PCR-enzyme-linked immunosorbent assay and partial rRNA gene sequencing: a rational approach to identifying mycobacteria. J. Clin. Microbiol., 35: 2375–2380.

Pazos, F., Santos, Y., Macías, A.R., Núñez, S. and Toranzo, A.E. 1996. Evaluation of media for the successful culture of *Flexibacter maritimus*. J. Fish Dis., 19: 193-197.

Pedersen, K., Kuhn, I., Seppanen, J., Hellstrom, A., Tiainen, T., Rimaila-Parnanen, E. and Larsen, J.L. 1999. Clonality of *Vibrio anguillarum* strains isolated from fish from the Scandinavian countries, Sweden, Finland and Denmark. J. Appl. Microbiol., 86: 337–347.

Petersen, A., Andersen, J.S., Kaewmak, T., Somsiri, T. and Dalsgaard, A. 2002. Impact of Integrated Fish Farming on Antimicrobial Resistance in a Pond Environment. Appl. Environ. Microbiol., 68: 6036-6042.

Plumb, J.A. 1999. Health maintenance and principle microbial diseases of cultured fishes. Iowa State University Press. Ames, Iowa: 344 pp

Prichard, R. 1997. Application of molecular biology in veterinary parasitology. Vet. Parasitol., 71: 155–175.

Pyle, S.W. and Shotts, E.B. 1980. A new approach for differentiating flexibacteria isolated from cold water and warmwater fish. Can. J. Fish. Aquat. Sci., 37: 1040-1042.

Rhodes, L.D., Rhodes, W.B. and Strom, M.S. 1998. Sensitive detection of *Renibacterium salmoninarum* in whole fry, blood, and other tissues of Pacific salmon by reverse transcription-polymerase chain reaction. Mol. Mar. Biol. Biotechnol., 7: 270–279.

Rithidech, K.N., Dunn, J.J. and Gordon, C.R. 1997. Combining multiplex and touchdown PCR to screen murine microsatellite polymorphisms. BioTechniques, 23: 36-45.

Sandaa, R.A., Torsvik, V.L. and Goksøyr, J. 1992. Transferable drug resistance in bacteria from fish-farm sediments. Can. J. Microbiol., 38: 1061-1065.

Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Pedersen, K. and Larsen, J.L. 2000. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. App. Environ. Microbiol., 66: 4908-4915.

Shalon, D.D., Smith, S.J. and Brown, P.O. 1996. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res., 6: 639–645.

Shewan, J.M. and McMeekin, T.A. 1983. Taxonomy and ecology of the *Flavobacterium* and related genera. Ann. Rev. Microbiol., 37: 233-252.

Southern, E.M. 1996. DNA chips: analysing sequence by hybridisation to oligonucleotides on a large scale. TIG, 12: 110–115.

Tijssen, P. 1993. Hybridisation with Nucleic Acid Probes, Elsevier, Amsterdam, 613 pp.

Tyagi, S. and Kramer, F.R. 1996. Molecular beacons: probes that fluoresce upon hybridisation. Nat. Biotechnol., 14: 303–308

Valsangiacomo, C., Baggi, F., Gaia, V., Balmelli, T., Peduzzi, R. and Piffaretti, J. 1995. Use of amplified fragment length polymorphism in molecular typing of *Legionella pneumophila* and application to epidemiological studies. J. Clin. Microbiol., 33: 1716-1719.

Vaneechoutte, M. 1996. DNA fingerprinting techniques for microorganisms. A proposal for classification and nomenclature. Mol. Biotechnol., 6: 115-142.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res., 23: 4407-4414.

Wegener, H.C., Aarestrup, F.M., Gerner-Smidt, P. and Bager, F. 1999. Transfer of antibiotic resistant bacteria from animals to man. Acta Vet. Scand. Supp., 92: 51-57.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., 18: 7213–7218.

Williams, K., Blake, S., Sweeney, A., Singer, J.T. and Nicholson, B.L. 1999. Multiplex reverse transcriptase PCR assay for simultaneous detection of three fish viruses. J. Clin. Microbiol., 37: 4139–4141.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol., 63: 3741–3751.

Zhou, J. and Dorothea K.T. 2004. Microarray technology and applications in environmental microbiology. Adv. Agronomy, 82: 183-270.

Zimmermann, K., Schogl, D., Plaimauer, B. and Mannhalter, J.W. 1996. Quantitative multiple competitive PCR of HIV-1 DNA in a single reaction tube. BioTechniques, 21: 480-484.

Zlotkin, A., Eldar, A., Ghittino, C. and Bercovier, H. 1998. Identification of *Lactococcus garvieae* by PCR. J. Clin. Microbiol., 36: 983–985.

Zou, S. 1997. A practical approach to genetic screening for influenza virus variants. J. Clin. Microbiol., 35: 2623-2627.