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Immunoserological and molecular techniques used in fish disease diagnosis- A mini review

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ABSTRACT
Immunoserological and Molecular techniques are potentially faster and more sensitive than culture 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, ELISA, in situ hybridization, and microarray. Pathogens can be detected from asymptomatic fish by either immunological or 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.

Keywords: Immunoserological, Molecular, Microarray, Asymptomatic.

1. Introduction
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.* [1]; Pazos *et al.* [2]). 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 [3]). Some attempts have been made using biochemical tests, DNA homology and protease variability (Pyle and Shotts [4]; Bertolini and Rohovec [5]; Chen *et al.* [6]), 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 (Plumb [7]) 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 (Altinok *et al.* [8]).

Fig 1: Commonly used disease diagnostic techniques

Immuno-serological and molecular techniques can be used to solve that type of problems and increase sensitivity and specificity of pathogen detection. These techniques include blotting techniques, enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization and in situ hybridization. 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.

2. Common Immunoserological and Molecular techniques used in disease diagnosis are

I. Gel Electrophoresis
II. Blotting techniques
III. Enzyme Linked Immunosorbent Assay (ELISA)
IV. Polymerase Chain Reaction (PCR)
V. Nucleic acid hybridization

a) Gel Electrophoresis

Gel electrophoresis is a basic technique used to separate DNA, RNA or proteins. It is a common starting point for many biotechnology experiments and is often paired with the blotting techniques.

This technique relies on electricity to separate out molecules in an agarose gel, a thick jelly-like substance. DNA, RNA and proteins are all electrically charged, so when an electric current is applied to the gel, these molecules will naturally move toward the opposite pole. Because the gel is difficult to travel through, the molecules will travel at different speeds depending on their size. Smaller molecules will be able to move faster and will reach the far end of the gel, while larger molecules will be slowed down and remain near the beginning.

The end result of gel electrophoresis is a gel with the molecules spread out from one end to the other. If they have been coloured, the molecules appear as short bands to the naked eye. The gel can be used in many different ways. Certain regions of the genome will result in a pattern that is unique for every person when run on a gel, which can be used for DNA fingerprinting. Gels are also used to detect the presence or absence of specific DNA or RNA molecules or proteins, when they are combined with the blotting techniques (Chamberlain *et al.* [9]).

b) The Blotting Techniques

The Southern, Northern and Western blots are used to detect DNA, messenger RNA (mRNA) and protein, respectively. Blotting refers to the actual technique, where molecules that have been separated on a gel are transferred or blotted onto a type of paper called nitrocellulose. The naming of the different blots originated with the DNA blot, developed by Edward Southern, and the Northern and Western blots followed.

I. Southern blotting

Before the blot itself can be done, DNA that has been cut up with restriction enzymes is separated by gel electrophoresis. For the blotting step, the gel is placed on a sponge which is sitting in a buffer solution. The nitrocellulose paper, where the DNA will be transferred to, is placed on top of the gel and then covered with paper towels and a weight. The transfer of the DNA from the gel to the paper happens by capillary action as the buffer moves toward the dry paper towels. After several hours, the transfer is complete and the paper will have the DNA fragments on it in the same pattern as they were in the gel. The paper can then be incubated with a probe that is specific to a DNA fragment of interest. The probe is radioactively labelled and once the incubation is complete, it can be detected by autoradiography. Controls must be used to ensure that the electrophoresis and the blot were successful. A comparison of the band patterns by autoradiography shows the presence or absence of the DNA of interest.

II. Northern blot

The northern blot is a technique used in molecular biology research to study gene expression by detection of RNA (or isolated mRNA) in a sample. With northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions. Northern blotting involves the use of electrophoresis to separate RNA samples by size and detection with a hybridization probe complementary to part of or the entire target sequence. The term 'northern blot' actually refers specifically to the capillary transfer of RNA from the electrophoresis gel to the blotting membrane. However, the entire process is commonly referred to as northern blotting. The northern blot technique was developed in 1977 by James Alwine, David Kemp, and George Stark at Stanford University. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot, named for biologist Edwin Southern. The major difference is that RNA, rather than DNA, is analyzed in the northern blot.

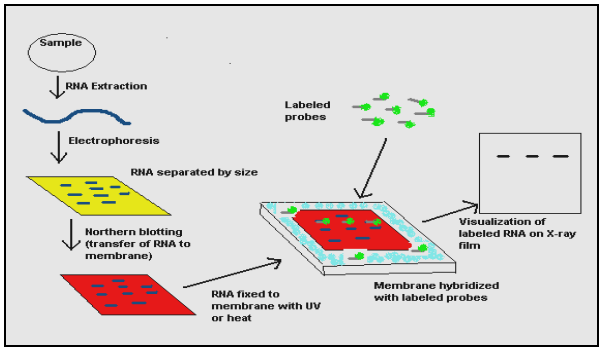

Fig 2: Northern blotting (http://en.wikipedia.org/wiki/Northern blot)

III. Western Blot

The western blot (sometimes called the protein immunoblot) is a widely accepted analytical technique used to detect specific proteins in the given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein.

There are now many reagent companies that specialize in providing antibodies (both monoclonal and polyclonal antibodies) against tens of thousands of different proteins.

Commercial antibodies can be expensive, although the unbound antibody can be reused between experiments. This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines.

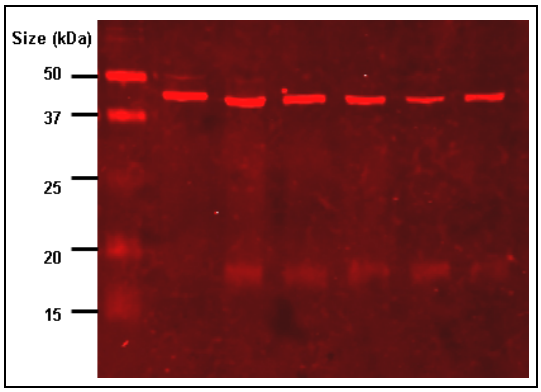

Fig 3: Western blots using an antibody that recognises protein (http://en.wikipedia.org/wiki/Western blot)

c) Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme linked immunosorbent assay (ELISA) is used for the detection and quantification of proteins typically secreted or released from cells. Immobilizing a target-specific capture antibody onto a high protein binding capacity ELISA plate enables capture of target protein. The captured protein is then detected by a protein-specific biotinylated antibody. The target protein is quantified using a colorimetric reaction based on activity of avidin-horseradish peroxidase (bound to the biotinylated detection antibody) on a specific substrate (e.g., ABTS, Super Aqua Blue or TMB). The optical density of the end-product is measured using a spectrophotometer. eBioscience offers a variety of ELISA to cytokines, chemokines, growth factors and protein released upon cell death.

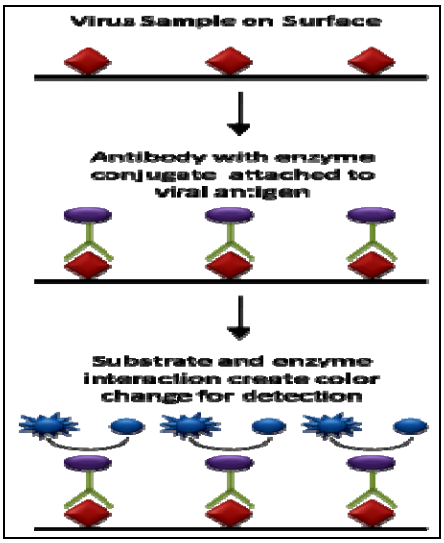

Fig 4: Direct ELISA

It is a very simple test that can analyse a large number of samples at once, which makes it a very important diagnostic technique. The ELISA method takes advantage of the natural property of antigens and antibodies to bond together. A plastic dish with many wells in it is coated with an antibody for a particular antigen. Then a different sample is added to each well - for example, blood samples from different people. Several wells will contain positive and negative control samples. If the antigens in the blood match the antibody in the well, they will bind. Those that do not bind will be washed off. A second antibody is then added to the wells, which will only attach to the antigens. This second antibody is attached to an enzyme ("enzyme-linked") that will produce a colour when a solution is added to it. The entire dish can then be read by a scanner that looks for the presence of the enzyme's colour. If a colour is present, it means that sample contained the antigen of interest. If there is no colour, there was no antigen to bind to the first antibody. The control samples are used to make sure the procedure was successful - the positive control should be coloured and the negative control should not.

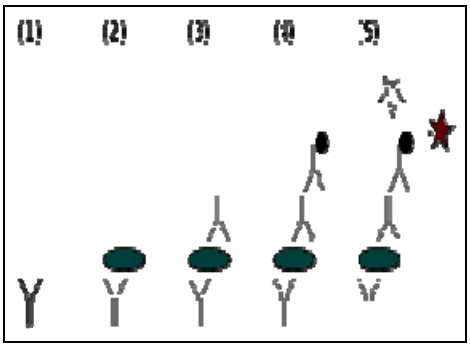

Fig 5: Sandwich ELISA (http://en.wikipedia.org/wiki/ELISA)

d) Polymerase Chain Reaction (PCR)

PCR is a technique used to make thousands of copies of a DNA strand in only minutes, using an enzyme called DNA polymerase. PCR plays an important role in research, diagnosis and forensics. To use PCR to amplify a DNA strand, the DNA sequences at both ends of the strand must first be known. Scientists can make complementary DNA of these regions, which are known as primers. The primers tell the DNA polymerase where to start copying the DNA and then when to stop. In addition to the primers, a copy of the DNA strand that needs to be copied, nucleotides and DNA polymerase are mixed in a small tube and put in a machine that can closely control the temperature. Specific changes in temperature are essential to the PCR process.

The starting temperature is 96 °C, which denatures, or separates the two strands of the DNA. The next step is called annealing, where the primers attach to the DNA strands. This happens at 68°C. Once the primers have annealed, DNA polymerase will extend them by adding nucleotides according to the DNA template at 72°C. Each cycle of these three steps takes less than two minutes and it can be repeated multiple times to produce thousands of copies of the original DNA strand.

PCR can be used for many purposes, including genetic fingerprinting in forensics, paternity testing, mutation detection for disease and cloning genes for research.

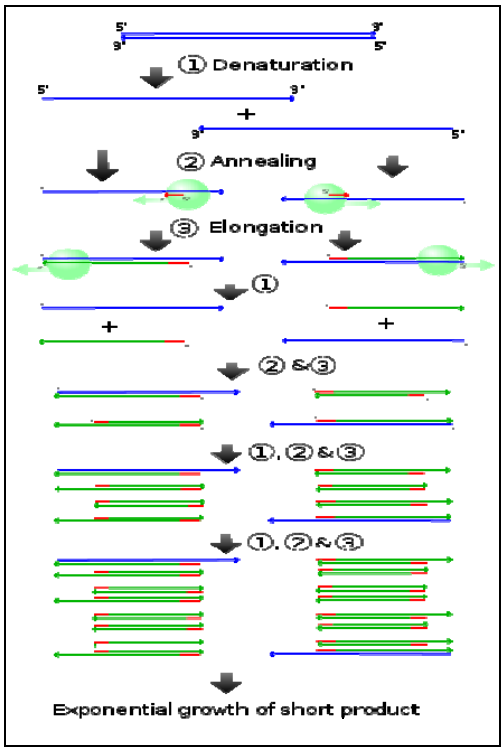

Fig 6: Schematic drawing of the PCR cycle (1) Denaturation at 94-96 ^{0}C. (2) Annealing at 65 ^{0}C (3) Elongation at 72 ^{0}C. Four cycles are shown here. The blue line represent the DNA template to which primers (red arrow) anneal that are extended by DNA polymerase (light green circle), to give shorted DNA products (green lines), which themselves are used as template as PCR progresses. (http://en.wikipedia.org/wiki/Polymerase chain reaction)

❖ **Methods to improve sensitivity of PCR**

- Addition of low concentration of dimethyl sulphoxide allows amplification of previously unamplifiable targets and allows larger target DNA to be amplified more efficiently.
- Addition of 20% glycerol allows amplification of upto 2500 bases.
- PCR specificity can be improved by "Hot start" method. If all the reaction mixtures are added at the same time and slowly heated at the start of PCR, the thermostable polymerase may extend any non-specific primer–template complex before denaturation can begin. This problem can be overcome by adding the polymerase after all the components have reached 70 ^{o}C.

❖ **Types of PCR**

A. Asymmetric PCR
B. Nested PCR
C. Multiplex PCR
D. Competitive PCR
E. Real-time PCR
F. RT-PCR

[1] RT-PCR

Since PCR can amplify dsDNA target sequences, single stranded RNA molecules cannot be amplified in the same way. Detection of mRNA is useful in detection of genes that are actively expressed. In order to detect viral nucleic acid (of RNA viruses) in clinical specimen as well as to prepare cDNA library of mRNA, a additional step is performed before the PCR cycles are initiated. Using the enzyme reverse transcriptase, a complimentary copy of the RNA is made. To generate cDNA using the enzyme reverse transcriptase, a primer is annealed to the template RNA. The primer can be gene specific primer, or oligo-dT primers can be used to initiate cDNA synthesis from mRNA. Using this primer, reverse transcriptase synthesizes cDNA strand by adding complementary base pairs. The template RNA is removed using the enzyme RNase H leaving behind the newly generated single stranded cDNA, which can be amplified in PCR. In the PCR reaction, one of the primers binds to the cDNA template and Taq polymerase extends the primer to produce the complementary strand. This results in the production of double stranded cDNA. It is then subjected to regular PCR cycles. Certain RT enzymes do not function beyond the non-stringent hybridization temperature of 42 ^{o}C. Single stranded RNA sometimes forms stable secondary structures and hamper conversion of RNA into cDNA. A thermostable RT along with raised temperature not only increases the stringency of hybridization but also disrupts any secondary structures that might have formed. A recombinant DNA polymerase derived from *Thermus thermophilus* (Tth pol) has both polymerase as well as RT activity in the presence of Mn^{2+}.

[2] Nested PCR

Nested PCR uses two sets of amplification primers. The first set of primers is used to amplify a target sequence and the second set of primers is used to amplify a region within the first target sequence. Essentially, this involves amplification of a sequence internal to an amplicon. Because the production of second amplicon depends on the successful production of the first amplicon, production of second amplicon automatically validates the accuracy of the first amplicon. Nested PCR may be performed in a single tube method or two-tubes method. In the single tube method, both the external and internal primer sets are added at the same time. There are two ways to accomplish nested PCR in single tube, one method involving physical separation of two sets of reaction and the other method involving difference in annealing temperature of primers. In physical separation method, the tube is filled with primary mixture consisting of target DNA, first (external) set of primers and other necessary components. This is overlaid with thick mineral oil layer into which second (external) set of primers and other components have been inserted. After the first round of 25-30 cycles of PCR, the reaction mixture is spun to mix the external primer and other components held inside the oil overlay. A second round of 25-30 cycles is performed and products are analysed.

In the differential annealing temperature method, the reaction mixture is set up to contain both the outer and inner sets of primer. These primer sets are so designed that outer primer pair hybridizes with the target at a temperature lower than what is required for hybridization of inner primer sets. Switching to higher temperature allows amplification of the internal product. In the two tube procedure, target is amplified using only the outer primer sets after 25-30 PCR cycles. After this, the tube is opened and the mixture is transferred to another tube containing inner primer sets which hybridizes to

the amplicon generated using outer primes. After running 25-30 cycles, the products are analyzed by standard methods.
Nested PCR makes the reaction very specific and alleviates false positive reactions that may occur with other PCR systems.

[3] Real time PCR

The "real-time" PCR system pioneered by Higuchi et al uses the intercalator ethidium bromide in each amplification reaction. An adapted thermal cycler is used to irradiate the samples with ultraviolet light, and the resulting fluorescence is detected with a computer-controlled cooled CCD camera. Amplification produces increasing amounts of double-stranded DNA, which binds ethidium bromide, resulting in an increase in fluorescence. By plotting the increase in fluorescence versus cycle number, the system produces amplification plots that provide quantitative picture of the PCR process. The principal drawback of intercalator-based detection of PCR product accumulation is that both specific and nonspecific products generate signal.
Subsequent improvements in Real-time systems for PCR were probe-based, rather than intercalator-based PCR product detection. The 5' nuclease assay provides a real-time method for detecting only specific amplification products. Cleavage of a target probe during PCR by the 5' nuclease activity of Taq DNA polymerase can be used to detect amplification of the target-specific product. In addition to the components of a typical amplification, reactions include a probe labeled with 32P on its 5' end and blocked at its 3' end so it cannot act as a primer. During amplification, annealing of the probe to its target sequence generates a substrate that is cleaved by the 5' nuclease activity of Taq DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. This dependence on polymerization ensures that cleavage of the probe occurs only if the target sequence is being amplified. After PCR, cleavage of the probe is measured by using thin layer chromatography to separate cleavage fragments from intact probe.
Quantification of the amount of target in unknown samples is accomplished by measuring CT and using the standard curve to determine starting copy number. The entire process of calculating CTs, preparing a standard curve, and determining starting copy number for unknowns is performed by the software. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the CT value. The early cycles of PCR are characterized by an exponential increase in target amplification. As reaction components become limiting, the rate of target amplification decreases until a plateau is reached and there is little or no net increase in PCR product. The sensitive fluorescence detection of the 5700 and 7700 systems allows the threshold cycle to be observed when PCR amplification is still in the exponential phase. This is the main reason why CT is a more reliable measure of starting copy number than an endpoint measurement of the amount of accumulated PCR product.

e) Nucleic Acid Hybridization

The two strands of a DNA molecule can be separated by exposing the DNA to high temperature, low salt or various chemicals. The process of denaturation or melting can be reversed by lowering the temperature, raising the salt concentration or removing the denaturation agent. The separated strands reassociate into double helix (duplex) and the process is known as renaturation or annealing. Since the hybridization requires sequence homology, a positive hybridization reaction between two nucleic acid strands each derived from different source indicate genetic relatedness between the two organisms. Hybridization assays require that one nucleic acid strand is from the known organism while the other is derived from the organism to be identified or detected (Southern [10])
If DNA from isolate obtained from a clinical specimen is mixed with a probe (labeled DNA) and denatured, the strands separate. Following reversal of the conditions, the probe strand would anneal with the isolate's strand if there is homology between the two. This reaction is called hybridization. The results of such experiments are expressed as percent hybridization/ percent similarity/ percent relatedness or D value.
Requirements for hybridization experiment include target nucleic acid (DNA/RNA), restriction endonuclease enzyme, labeled probes, polyacrylamide gel/ agarose electrophoresis apparatus, nylon/nitrocellulose membrane and stringent conditions.

Steps involved in hybridization reactions are:

- Production and labeling of single stranded probes
- Preparation of single stranded target nucleic acid
- Mixture of target and probe to allow annealing
- Detection of hybridization reaction

Probes are short nucleic acids with known nucleotide sequences designed to hybridize with the target nucleic acid. Probes are labeled to enable their detection after hybridization. To synthesize a probe against a target sequence, the nucleotide sequence of the target must be known. Probes are prepared against target sequences that are unique to a given organism or a group of organism or a virus to prevent non-specific binding (Tijssen [11])

Probes are prepared using one of these methods:

a. Cloning on vectors such as plasmids, λ phages, YACs or Cosmids
b. Chemical synthesis of oligonucleotide probes (~ 20 nucleotides)
c. PCR amplification of known sequence

Probes can vary in length, they can be short oligonucleotides (20-40 nucleotides) or cDNA probe of 1500-3000 bp in length. Oligonucleotide probes are convenient because they can be synthesized in large quantities artificially and their short length allows for highly specific discrimination of single nucleotide changes in hybridization reactions. Oligonucleotide probes are preferred in in-situ hybridization because their compact size allows them to penetrate the tissue better than larger probes. However, shorter probes have some limitations too; shorter the probe the more likely it is find a closely - 5 - similar sequences within target DNA. This may result in background cross hybridization and false positive hybridization results. cDNA probes are thus more specific than oligonucleotide probes.
The probes are labeled to facilitate their detection following hybridization to their target sequence. Once the probe is ready

it must be labeled with a signal generating moiety. Labeling can be done using radioactive or non-radioactive labels. Signal generating tags are also called reporter molecules.

The common radioactive isotopes used for labeling include ^{32}P, ^{35}S and ^{125}I. These isotopes are tagged to the nucleotides of the probe by techniques such as nick translation or random priming. Once hybridized, the labeled probes can be detected by scintillation counter or on X-ray autoradiography. Even though radioactive isotope labels provide maximum sensitivity, their disadvantages include higher expense, difficulty in handling, health hazard, short shelf life and disposal issues.

i. In Situ Hybridization

In order to obtain microbial nucleic acid for hybridization, the nucleic acid extraction technique destroys the tissue and the histopathological architecture is lost. Sometimes, it is very significant to localize the microbial nucleic acid in the tissue section. Both the DNA and RNA in routine - 9 - histopathologic tissue sections remain intact and accessible for hybridization even after fixation in formalin and embedding in hot paraffin. In in-situ hybridization the microscopy glass slide acts as the solid phase.

The stages of in situ hybridization are deparaffinization of tissue section, protease digestion to expose nucleic acid targets, post fixation in paraformaldehyde, application of probe, denaturation of DNA at high temperature, hybridization at physiological temperature, washing and detection of signals. Radio-labeled probes and biotinylated probes are commonly used. Using this method it is possible to perform molecular hybridization while simultaneously viewing the histopathological structure of the specimen. This technique allows precise detection of infected cells in the tissue. Information provided by this assay can confirm the target organism's role in the infectious process as well as give additional information on its distribution and abundance. This technique is useful in detecting intracellular parasites such as viruses and malignancies. Since the probe has to reach the target inside the cells, only probes that are small (~300 bases) can be used for tissue penetration, hence sensitivity is limited to the accessibility of the target in the cell.

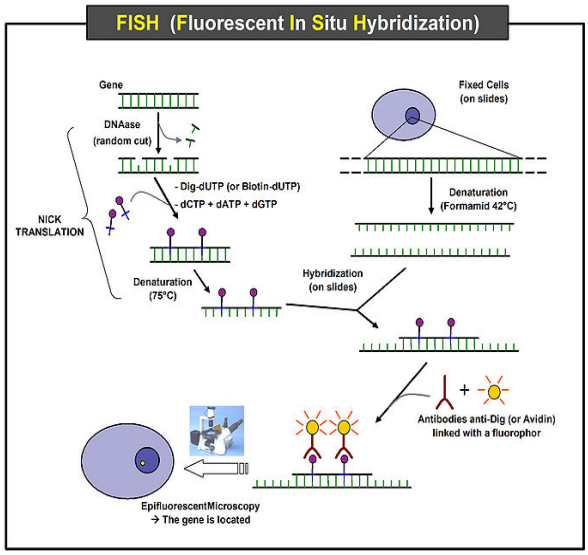

Fig 7: Fluorescent in Situ Hybridization (FISH) (http://en.wikipedia.org/wiki/Fluorescence in situ hybridization [12])

ii. Dot or slot hybridization

Nucleic acid which is present in clinical samples in reasonable number (104-105) molecules/ml can be readily and specifically detected by dot of slot hybridization. DNA of the organisms in the clinical specimen are lysed to obtain their DNA, denatured to separate the strands and transferred on to nylon membranes in a dot or slot fashion and fixed. The nucleic acids on the nylon membrane are single stranded and can bind to labeled probes. The membrane is immersed into a solution containing labeled probes and allowed to hybridize. Unbound probes are washed away and the hybridized duplexes are detected according to the nature of the reporter molecules. The advantages of this technique are that a single membrane can be used to test several specimens and a single specimen can be tested for several organisms on the same membrane.

3. Applications of immunological and molecular techniques in disease diagnosis

- ✓ Detection of pathogenic microorganisms in a mixture
- ✓ Detection of organisms that have become non-viable
- ✓ Detection of organisms that cannot be cultured or difficult to grow
- ✓ Rapid detection of organisms that grow slowly
- ✓ Detection of previously unknown (novel) organisms
- ✓ Identification and classification of novel isolates
- ✓ Quantification of infectious agent burden; of significance in monitoring disease
- ✓ Detection of antimicrobial resistance
- ✓ Detection of organisms for which reliable diagnostic methods are not available
- ✓ Characterization of microorganisms beyond identification
- ✓ Investigation of strain relatedness for epidemiological typing
- ✓ Differentiation of toxigenic from non-toxigenic strains.
- ✓ Detection of microbial virulence factors
- ✓ Diagnosis in animal where serological markers are unreliable
- ✓ Differentiation of pathogenic from non-pathogenic isolates
- ✓ Detection of contaminating viruses in tissue culture
- ✓ Synthesis of oligonucleotide probes in large numbers by PCR
- ✓ Exact localization of virus infection or tumors in tissue by in-situ hybridization
- ✓ Identification of etiology where multiple organisms can cause similar conditions
- ✓ Detection of mutations and base pair changes
- ✓ Differentiation of wild-type from vaccine strains
- ✓ Diagnosis of congenital infections
- ✓ Applications in HLA typing, anthropology, disputed paternity etc. are some of the other applications. (Sridhar [13]).

4. Conclusion

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. Acknowledgement
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