

Advanced Technology for the Diagnosis of Fish Diseases

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Abstract: Nucleic acid-based testing is becoming a crucial diagnostic tool not only in the setting of inherited genetic disease but also in a wide variety of infectious conditions. Molecular diagnostics provides the necessary underpinnings for any successful application. It offers a great tool for assessing therapy response and detecting minimal residual disease. During the last decades or so, molecular techniques have been increasingly employed to diagnose fish diseases.

Molecular techniques are potentially faster and more sensitive than culture, serology, and histopathological methods that are traditionally used to identify fish pathogens.

In this paper we reviewed briefly and discussed, the advanced techniques used for molecular diagnosis of fish diseases

Keywords: Advanced techniques, Fish Diseases ,Diagnosis, Application.

1. Introduction

Fishes, as with other animals, are subjected to a wide spectrum of diseases. Such diseases require a somewhat different approach to solving the problem than terrestrial animals.

All forms of aquaculture are susceptible to outbreaks of disease, as many pathogenic bacteria are normal inhabitants of the aquatic environment. Both in aquaculture facilities and in external aquatic environments, the occurrence of disease is a complex interaction between the host species, disease agents and the environment. In farm environments, outbreaks of disease are greatly influenced by the susceptibility of the hosts, the virulence of the pathogens and adverse environmental conditions. Farming practices may favor disease occurrence, as in the case of intensive and semi-intensive systems of production characterized by high stocking densities, increased stress of stocks and inadequate water exchange. As a result, it is not uncommon for outbreaks of epizootics to occur in aquaculture farms. Aquaculture literature describes a large number of infectious diseases caused by various pathogenic agents. On the other hand, disease outbreaks are less common in open water environments, even though the pathogens and host species may be present.

Several advanced technologies are now being adopted for better health management practices in aquaculture, with prevention and control of fish diseases. The modern diagnostic assays are based on interactions of certain

important biomolecules of the pathogens such as antigen, nucleic acids etc. following their isolation, purification and characterization for preparing suitable diagnostic and/or prophylactic tools. Enzymes, radioisotopes and fluorescent dyes are used in certain advanced serological techniques like enzyme-linked immunosorbent assay (ELISA), western blotting, radioimmunoassay and fluorescent antibody test. Several ELISA systems employing monoclonal antibodies to fish pathogens have been described.

Proper diagnosis leads to accurate therapies and avoid indiscriminate use of chemotherapeutics. Pathogens can be detected from asymptomatic fish by molecular diagnostic techniques so disease outbreak could be prevented.

2. Methodology

Immunological techniques

Are used for diagnosis of various diseases or to study the expression profile of various genes:

Western blotting
Immunofluorescence
Immunoperoxidase/enzyme immunohistochemistry
Flowcytometry
Confocal laser microscopic techniques

Western blotting

Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of

proteins extracted from cells. The technique (fig.1) uses three elements to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody to visualize. This paper will attempt to explain the technique and theory behind western blot, and offer some ways to troubleshoot.

Fig.1: Western blotting.

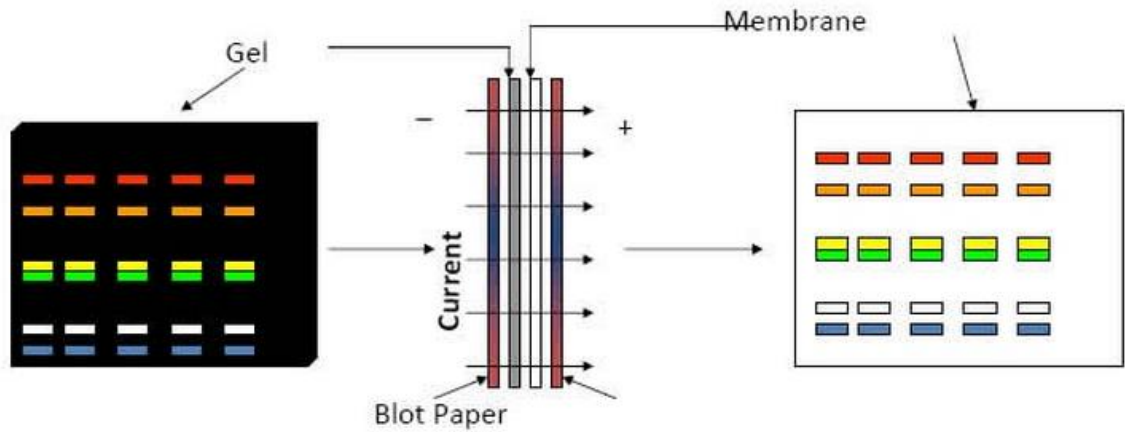
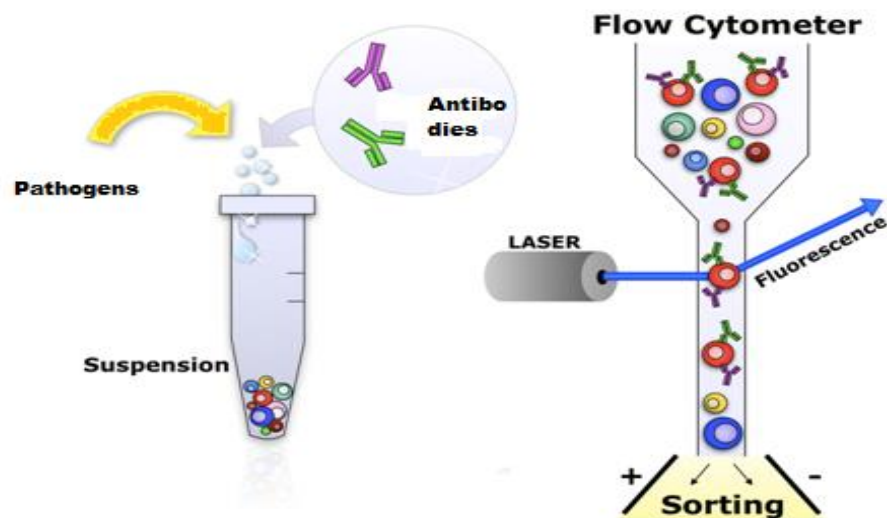


Fig. 2: Confocal laser microscopic techniques



Fig.3: Flowcytometry



Fluorescent antibody technique

Either of two techniques used to test for antigen with a fluorescent antibody: direct, in which immunoglobulin conjugated with a fluorescent dye is added to tissue and combines with a specific antigen; or indirect, in which unlabeled immunoglobulin is added to tissue and combines with a specific antigen, after which the antigen-antibody complex may be labeled with a fluorescent antibody.

ELISA, or EIA, is an acronym for enzyme-linked immune-assay. ELISA is a test that detects and measures antibodies in your blood. This test can be used to determine if you have antibodies that are related to certain infectious conditions. Antibodies are proteins that the body produces in response to harmful substances (antigens). ELISA is a popular format of "wet-lab" type analytic biochemistry assay that uses a solid-phase **enzyme immunoassay (EIA)** to detect the presence of a substance, usually an antigen, in a liquid sample or wet sample.

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are non-specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

Fig. 4: The various components required for PCR include a DNA sample, DNA primers, free nucleotides called ddNTPs, and DNA polymerase.



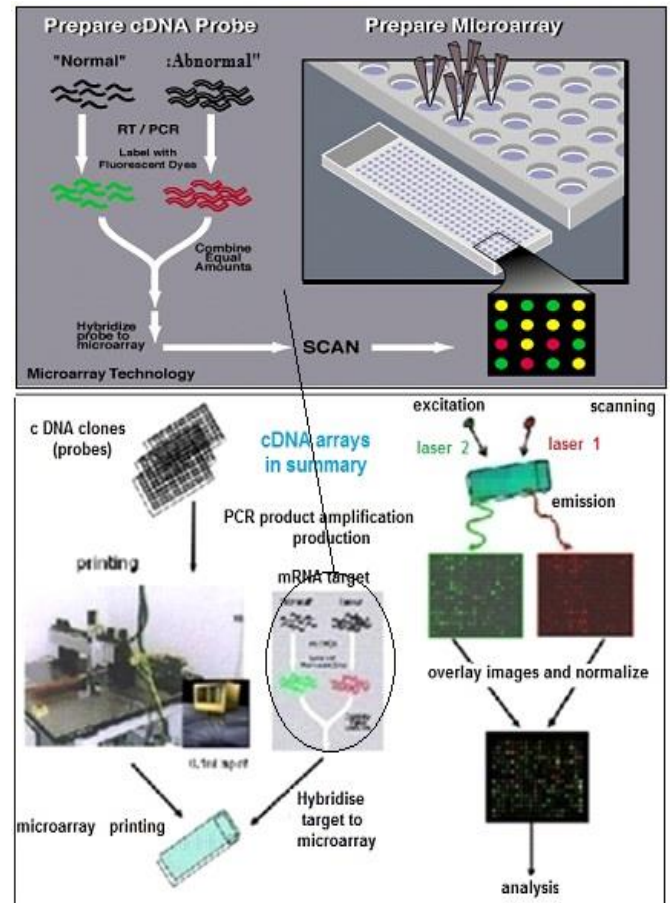
Molecular diagnostic methods based on detection of Nucleic Acids

- Polymerase chain reaction (PCR) - Real time PCR .
 - DNA probes.
 - Amplified Fragment Length Polymorphism (AFLP)
 - DNA Based Typing of Microorganisms:
 1. Random Amplification of Polymorphic DNA (RAPD)
 2. Restriction fragment length polymorphisms (RFLP)
 3. PCR-RFLP
 4. Pulsed field gel electrophoresis (PFGE)
- Polymerase Chain Reaction

Fig. (5-1and 5-2) DNA microarrays, as a valuable tool for identification and diagnosis of fish diseases.

Confocal laser microscopic techniques

A number of different imaging modes are used in the application of confocal microscopy to a vast variety of specimen types. They all rely on the ability of the technique to produce high-resolution images, termed **optical sections**, in sequence through relatively thick sections or whole-mount specimens.



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. Primer design is important to obtain greatest possible sensitivity and specificity.

PCR is extremely efficient and sensitive; it can make millions or billions of copies of any specific sequence of DNA, even when the sequence is in a complex mixture. Because of this power, researchers can use it to amplify sequences even if they only have a minute amount of DNA. A single pathogen,

or a microscopic blood stain for example, contains ample DNA for PCR [1, 2].

In PCR, a DNA sequence that a researcher wants to amplify, called the "target" sequence, undergoes about thirty rounds of replication in a small reaction tube. During each replication cycle, the number of molecules of the target sequence doubles, because the products and templates of one round of replication all become the templates for the next round. After n "rounds of replication, 2^n copies of the target sequence are theoretically produced. After thirty cycles, PCR can produce 2^{30} or more than ten billion copies of a single target DNA sequence. This is called a polymerase chain reaction because DNA polymerase catalyzes a chain reaction of replication.

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 and has been shown to be rapid, reproducible, and highly discriminatory. 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 [5]. 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. 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 *EcoRI*) and a second one with a higher cutting frequency (like *MseI* or *TaqI*). 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. Alternative AFLP typing procedures are based on one enzyme with a single adapter and analysis by agarose gel electrophoresis. A major improvement has been obtained by switching from radioactive to fluorescently labeled primers for detection of fragments in an automatic sequence. 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 is sufficient.

Hybridization methods

The term "hybridization" refers to the chemical reaction between the probe and the DNA or RNA to be detected. If hybridization is performed on actual tissue sections, cells, or isolated chromosomes in order to detect the site where the DNA or RNA is located, it is said to be done "*in situ*." By

contrast, "*in vitro*" hybridization takes place in a test tube or other apparatus, and is used to isolate DNA or RNA, or to determine sequence similarity of two nucleotide segments.

In situ hybridization is a technique used to detect specific DNA and RNA sequences in a biological sample [6]. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are macromolecules made up of different sequences of four nucleotide bases (adenine, guanine, uracil, cytosine, and thymidine). *In situ* hybridization takes advantage of the fact that each nucleotide base binds with a complementary nucleotide base. For instance, adenine binds with thymidine (in DNA) or uracil (in RNA) using hydrogen bonding. Similarly, guanine binds with cytosine. In a specialized molecular biology laboratory, researchers can make a sequence of nucleotide bases that is complementary to a target sequence that occurs naturally in a cell (in a gene, for example). When this complementary sequence is exposed to the cell, it will bind with that naturally occurring target DNA or RNA in that cell, thus forming what is known as a hybrid. The complementary sequence thus can be used as a "probe" for cellular RNA or DNA.

Random Amplified Polymorphic DNA (RAPD)

The technically demanding method of RAPD has been applied to the study of crayfish plague fungus, *Astacus astaci*. RAPD uses a single primer in low-stringency polymerase chain reactions. Random binding of primers results in different sizes of fragments from samples with non-identical DNA. Application of the RAPD technique grouped different isolates of the fungus and provides the means to carry out epidemiological investigations [7, 8]. 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 [7]. 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 [9, 10] 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.

DNA probes

Gel electrophoresis is a method used in clinical chemistry to separate proteins by charge and/ or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge [11]. Nucleic acid molecules are separated by applying an electric field [12] to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving [12]. Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins. Gel electrophoresis can also be used for separation of nano-particles.

Gel electrophoresis uses a gel as an anticonvective medium and or sieving medium during electrophoresis, the movement

of a charged particle in an electrical field. Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of molecules; gels can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied [13]. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting [14] for further characterization.

Application of the Probe for DNA or RNA to Tissues or Cells: *In situ* hybridization allows us to learn more about the geographical location of, for example, the messenger RNA (mRNA) in a cell or tissue. It can also tell us where a gene is located on a chromosome. Obviously, a detection system must be built into the technique to allow the cytochemist to visualize and map the geography of these molecules in the cells in question.

When *in situ* hybridization was first introduced, it was applied to isolated cell nuclei to detect specific DNA sequences. Early users applied the techniques to isolated chromosomal preparations in order to map the location of genes in those chromosomes. The technique has also been used to detect viral DNA in an infected cell.

Innovative molecular tools

Multiplex PCR

Multiplex PCR is new development, 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 [15], countering one of the major arguments against the adoption of these techniques as routine. 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, quantitative and RNA detection. In the field of infectious diseases, the technique has been shown to be a valuable

method for identification of viruses, bacteria, fungi and parasites.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions [18, 19, 20, 21]. When combined with reverse transcription, this method can also amplify RNA sequences with high efficiency. The method relies on auto-cycling strand displacement DNA synthesis using a DNA polymerase with a high strand displacement activity and a set of four specially designed primers. These four primers, termed as inner and outer primers, recognize six distinct sequences of the target DNA, which improves the specificity of the reaction. The reaction is carried out at isothermal condition, as the denaturation of strands takes place by strand displacement.

DNA Microarrays

Gene expression profiling using DNA microarrays holds great promise for the future of molecular diagnostics [16]. This technology allows, in one assay, for simultaneous assessment of the expression rate of thousands of genes in a particular sample. The 2 types of DNA microarrays that are widely used are cDNA microarrays and oligonucleotide/DNA chips [17].

In cDNA microarrays (fig.5-1), DNA sequences complementary to a library of mRNA from thousands of genes are mechanically placed on a single glass slide. The immobilized cDNA sequences serve as anchoring probes to which mRNA extracted from the tested sample will specifically attach during hybridization. If the tested mRNA is first tagged with a fluorescent dye, the intensity of fluorescence at each anchoring probe location will be proportional to the amount of mRNA (degree of expression) of the gene at that location. A microarray reader displays the intensity of fluorescence at each cDNA location as a colored dot per gene location on a grid (fig.5-2). In the field of infectious diseases, microarrays has been shown to be a valuable method for identification and diagnosis of fish diseases caused by viruses, bacteria, fungi and protozoa in one step.

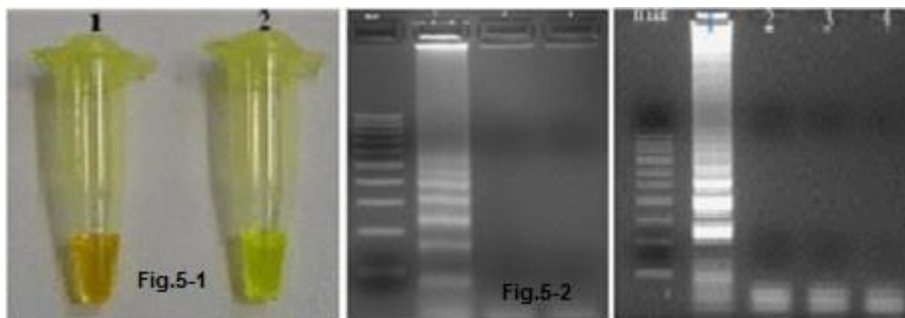


Fig.(5-1): Visual detection of KHV LAMP products using SYBR Green I stain. 1: negative LAMP reaction remained orange. 2: positive LAMP reaction turned green. **Fig.(5-2):** Agarose gel showing LAMP products of KHV DNA extracted by boiling. The reaction was carried out at 65°C using the 6 primer set. Lanes: mar = 100 bp molecular weight marker; 1 = KHV DNA extracted by boiling; 2 = negative fish tissue; 3 = negative control. Agarose gel illustrating the specificity of the designed primers to KHV DNA. The reaction was carried out at 65°C using the 6 primer set for 1 hr. Lanes: 1 = KHV DNA; 2 = *Herpes*

virus cyprini (CHV) DNA showing no amplification; 3 = channel catfish virus (CCV) showing no amplification; 4 = uninfected koi tissue; mar = 100 bp DNA molecular weight marker [21].

The test is including the following:

1. **LAMP Reaction:** In the initial stages of LAMP reaction all four primers are involved, however, in the later cycling reaction only the inner primers are used for strand displacement DNA synthesis. The LAMP reaction is initiated by an inner primer containing sequences of sense and anti-sense strands of the target DNA. This is followed by the release of a single-stranded DNA through the priming by an outer primer. This single-stranded DNA will serve as a template for DNA synthesis primed by the second inner and outer primers that can hybridize at the other end of the target. This process will result in the formation of a stem-loop DNA structure. In the subsequent step of LAMP cycling, one inner primer will hybridize to the loop on the product and initiate strand displacement DNA synthesis which will result in the original stem-loop DNA and a new stem-loop. Cycling continues for a period of approximately 1 h and results in the accumulation of 10^9 copies the target. The final products of the reaction are stem-loop DNA with several inverted repeats of the target and cauliflower-like structures with multiple loops.
2. **Visualization of Amplified Products:** Several methods can be employed to visualize the end products of LAMP reaction. The most common method of visualization is by agarose gel electrophoresis. The agarose gel is stained with intercalating dyes such as ethidium bromide or SYBR Green I. Since the end products of LAMP consist of stem-loop DNA and cauliflower-like structures with multiple loops of various lengths, the agarose gel electrophoresis will reveal the products from the minimum length of the target DNA to the loading well, which appears as smear at the top and bands at the base of the gel. Since, one of the characteristics of the LAMP reaction is its ability to synthesize extremely large amount of DNA, addition of intercalating dye, SYBR Green I, into the reaction tube itself would help in visualizing the product under a UV- transilluminator. This method is useful in the field-level application, where gel electrophoresis will be a limiting factor. Another method is also based on the accumulation large amount of byproduct of the reaction. In the LAMP reaction large amount of by product, pyrophosphate ion is produced, which will yield white precipitate of magnesium pyrophosphate in the reaction mixture. Hence, detection of presence or absence of white precipitate will provide an easy distinction of whether the target DNA is amplified during the reaction. Further, since increase in the turbidity of the reaction mixture according to the production of the precipitate correlates to the amount of target DNA synthesized, a colorimetric estimation of the turbidity in real-time is also being used as an efficient method of visualizing the amplified product.

Advantages of LAMP: LAMP amplifies the target DNA under isothermal amplification with high efficiency; The detection limit of LAMP is a few copies and comparable to PCR; No significant influence of the co-presence of non-target DNA; LAMP allows simple, easy and selective detection; Lamp is highly specific for the target sequence, as it employs four primers targeting multiple sequences; LAMP is simple and easy to perform, as it requires (after appropriate

primers are prepared) only a regular laboratory water bath or heat block for the reaction; BY incorporating reverse transcription, LAMP can be used for amplifying RNA as well. Disadvantages: Because amplification of the target DNA is so high at the final stage it is vulnerable to contamination in subsequent amplifications; Multiplexing is not possible with LAMP.

Bluspot Technology

Bluspot technology is a universal membrane-based detection technique which combines an immunological and enzymatic reaction and may address the market of laboratories that don't have automated testing equipment [22]. Blusopt technology can be used as a platform for development of rapid tests in high potential and/or niche applications (e.g. environment and fish sector, and detection of allergens).

The technology is based on filtration (flow through) of reagents through a membrane on which a capture agent specific of the molecule to be detected is bound. In case of a positive result, an enzymatic reaction leads to the formation of a precipitated blue spot (fig.6) within 5 minutes.

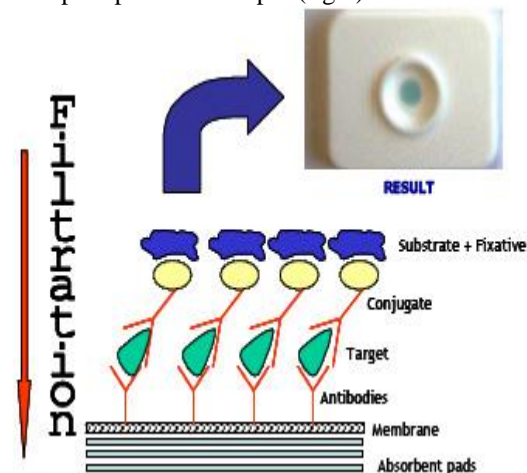


Fig.6 BluspotTechnology The sample to be tested (e.g. water, blood, serum, tissue, environmental sample) migrates through a membrane on which specific antibodies are coated. Antigen-antibody complexes are revealed by the use of a “conjugate” enzyme. After addition of an insoluble precipitating substrate, and a stopping solution, a permanent blue colored spot is obtained, for which the intensity is proportional to the concentration of the target. As a result, the method does not only allow accurate detection, but also quantification of results may be interpreted visually (qualitative or semi-quantitative interpretation by comparison with a reference color chart) or by the use of a reflectance reader (quantitative determination).

Nanotechnology

Nanotechnology is broadly defined as systems or devices related to the features of nanometer scale (one billionth of a metre). The small dimensions of this technology have led to the use of nanoarrays and nanochips as test platforms [23]. One advantage of this technology is the potential to analyze a

sample for an array of infectious agents on a single chip. Applications include the identification of specific strains or serotypes of disease agents or the differentiation of diseases caused by different viruses but with similar clinical signs. Another facet of nanotechnology is the use of nanoparticles to label antibodies. The labeled antibodies can then be used in various assays to identify specific pathogens, molecules or structures. Example of nanoparticle technology includes the use of gold nanoparticles, nanobarcodes, quantum dots and nanoparticle probes [24]. Additional nanotechnologies include nanopores, cantilever arrays, nanosensors and resonance light scattering. Nanopores can be used to sequence strands of DNA as they pass through an electrically-charged. Nanotechnology is still in the research stage but it is anticipated that nanotechnologies will eventually be applied to the diagnosis of endemic veterinary diseases in the future.

Biosensor

A new approach to the detection of either the agent or antibodies is the development of biosensors. This type of assay involves the use of a receptor (usually an antibody) for the target pathogen or a disease-specific antibody and a transducer which converts a biological interaction into a measurable signal [25]. Some of the transducer technologies under development include electrochemistry, reflectometry, interferometry, resonance and fluorimetry. Biosensors are frequently coupled to sophisticated instrumentation to produce highly-specific analytical tools, most of which are still in use only for research and development due to the high cost of the instrumentation, the high cost of individual samples analysis, and the need for highly trained personnel to oversee the testing.

A biosensor typically consists of a bio-recognition component, biotransducer component, and electronic system which include a signal amplifier, processor, and display. Transducers and electronics can be combined, e.g., in CMOS-based microsensor systems. [25]. The recognition component, often called a bioreceptor, uses biomolecules from organisms or receptors modeled after biological systems to interact with the analyte of interest. This interaction is measured by the biotransducer which outputs a measurable signal proportional to the presence of the target analyte in the sample. The general aim of the design of a biosensor is to enable quick, convenient testing at the point of concern or care where the sample was procured.

In a biosensor, the bioreceptor is designed to interact with the specific analyte of interest to produce an effect measurable by the transducer. High selectivity for the analyte among a matrix of other chemical or biological components is a key requirement of the bioreceptor. While the type of biomolecule used can vary widely, biosensors can be classified according to common types bioreceptor interactions involving: antibody/antigen, enzymes, nucleic acids/DNA, cellular structures/cells, or biomimetic materials.

Microfluidic approach

A plug-based microfluidic approach (fig.8) combining the technique of the chemistode and the principle of stochastic confinement, which can be used to: i) starting from a mixture of cells, stochastically isolate single cells into plugs, ii) incubate the plugs to grow clones of the individual cells without competition among different clones, iii) split the plugs into arrays of identical daughter plugs, where each plug contained clones of the original cell, and iv) analyze each array by an independent technique, including cellulase assays, cultivation, cryo-preservation, Gram staining, and Fluorescence *In Situ* Hybridization (FISH). Functionally, this approach is equivalent to simultaneously assaying the clonal daughter cells by multiple killing and non-killing methods. A new protocol for single-cell FISH, a killing method, was developed to identify isolated cells of *Paenibacillus curdlanolyticus* in one array of daughter plugs using a 16S rRNA probe, Pc196. At the same time, live copies of *P. curdlanolyticus* in another array were obtained for cultivation. Among technical advances, this paper reports a chemistode that enables sampling of nanoliter volumes directly from environmental specimens, such as soil slurries. In addition, a method for analyzing plugs is described: an array of droplets is deposited on the surface, and individual plugs are injected into the droplets of the surface array to induce a reaction and enable microscopy without distortions associated with curvature of plugs. The overall approach is attractive for identifying rare, slow growing microorganisms and would complement current methods to cultivate unculturable microbes from environmental samples [26].

Fluorescent in situ hybridization (FISH)

FISH (fig.9) is a technique that can localize nucleic acid sequences within cellular material. Peptide nucleic acids, molecules in which the sugar backbone has been replaced by a peptide backbone, are perfect mimics of DNA with high affinity for hybridization that can be used to improve FISH techniques [27].

3. Conclusion

Apart from the sensitivity and rapidity of diagnosis, principal advantage of molecular and technological diagnostic methods is in the detection of non-culturable agents; DNA amplification can assist in detecting the pathogens that are present in low numbers and also in handling a tiny volume of specimen; can be used to detect latent infection and thereby identify the reservoir hosts of infection that is significant in epizootiology; can be used to differentiate antigenically similar pathogens.

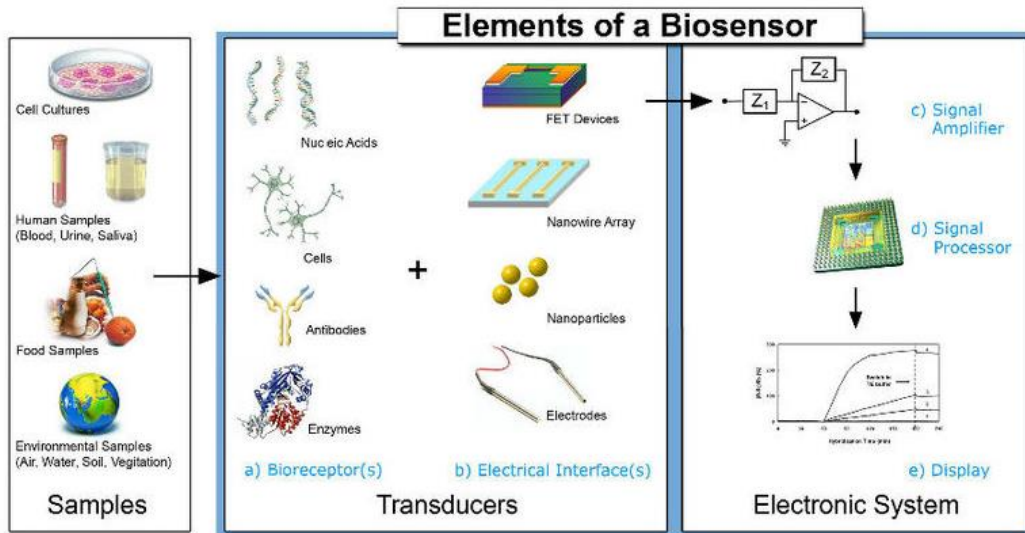
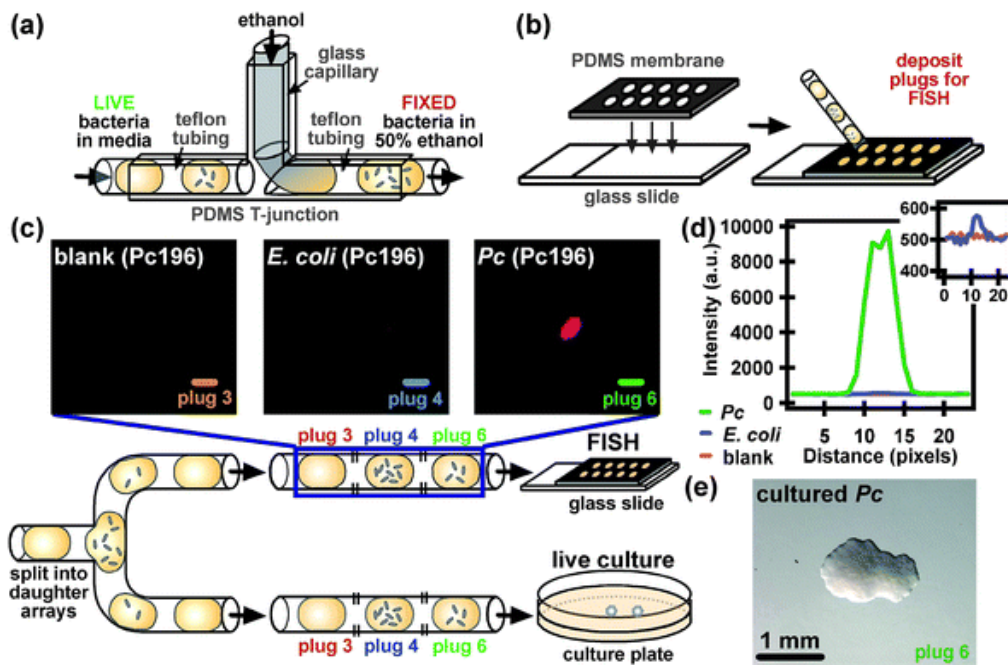


Fig.7:A biosensor is an analytical device, used for the detection of an analyte, that combines a biological component with a physicochemical detector. [25].



A. Fig.8:Isolation, incubation, and parallel functional testing and identification by FISH of rare microbial single-copy cells from multi-species mixtures using the combination of chemistride and stochastic confinement[26].

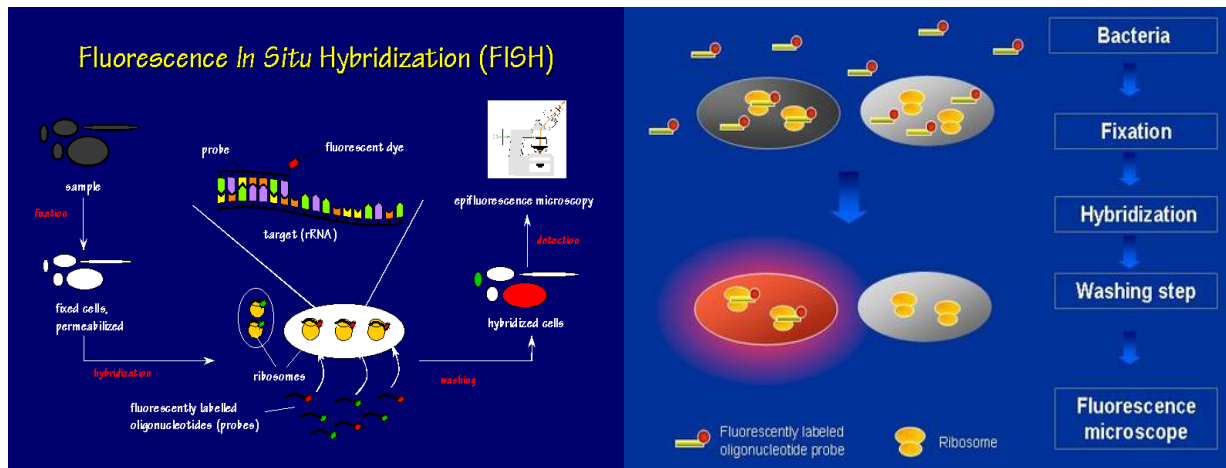


Figure 9 . Fluorescent *In Situ* Hybridization Flow Chart. The samples are fixed onto coverslips, fluorescently labeled oligonucleotide probes are hybridized with the sample, excess probes are washed away, and epifluorescence microscopy is used to view the location of the hybridized cells [28].

Conflict of interest

The author has declared no conflict of interest.

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