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CHAPTER TEN

Immunological and molecular biology techniques in disease diagnosis

Leobert D. de la Peña

Traditionally, the diagnosis of infectious diseases has been accomplished by the isolation of the infecting microorganism in pure culture. Classical methods of microbial isolation and identification have been invaluable in the study of bacterial, viral and fungal infections. However, cultivation systems offer disadvantages for the rapid diagnosis of infectious diseases. For example, many microorganisms, especially viruses and slow growing bacteria, require a considerable period of time in cultivation that the results from cultures are often not available at a time when the result can alter the course of therapy. Thus, more sensitive means must be applied for detecting and identifying a wide range of infectious diseases of fish and shrimps.

The development of rapid, simple, sensitive and specific diagnostic tests for infectious diseases has been much slower than the other disciplines of medicine. This is partially attributed to the complexity and diversity of the pathogenic organisms and to the difficulty in detecting low concentrations of these organisms in samples. Immunological and molecular biology-based techniques are rapidly advancing the field of diagnostics in fish and shrimp diseases.

IMMUNOLOGICAL TECHNIQUES

Antigen-antibody reactions are most easily studied *in vitro* using preparations of antigens and antisera. Reactions of antigens and antibodies are highly specific. An antigen will react only with antibodies elicited by itself or by a closely related antigen. Because of the high specificity, reactions between antigens and antibodies are suitable for identifying one by using the other. However, cross-reactions between related antigens can occur, and these can limit the use-fulness of the test. The study of antigen-antibody reactions *in vitro* is called serology, and is important in clinical diagnostic microbiology.

Immunodiagnostic tests use an antigen-antibody reaction to detect and identify a specific antigen or antibody associated with a disease-causing organism. The antigen-antibody reaction itself is very specific. Consequently, if the correct antibodies can be obtained, immunodiagnostic techniques have the advantage of being able to identify the presence of a specific pathogen directly in the

specimens and also can be used to detect the specific antibodies produced as a result of the immune response of the host to the organism. The primary component in the test is the antibody. The specificity, and to some extent the sensitivity, of the assay depends on the quality of the antibodies used in the reagents.

Antibodies are formed by clonal selection as explained in Chapter 9. A large pool of B lymphocytes (B cells) display immunoglobulin (Ig) molecules on their surface. These Ig serve as receptors for a specific antigen, so that each B cell can respond to only one antigen or a closely related group of antigens. An antigen interacts with the B lymphocyte that shows the best "fit" by virtue of its Ig surface receptor. The antigen binds to this receptor, and the B cell is stimulated to divide and form a clone. Such selected B cells soon become plasma cells and secrete antibody. Since each person can make 10^7-10^8 differ-ent antibody molecules, there is an antigen-binding site on a B cell to fit almost any antigenic determinant.

Antibodies are immunoglobulins that reacts specifically with the antigen that stimulated their production. Antibodies that arise in an animal in response to a single antigen are heterogeneous because they are formed by several different clones of cells; i.e., they are polyclonal antibodies. Antibodies that arise from a single clone of cells, e.g., in a plasma cell tumor (myeloma), are homogeneous; i.e., they are monoclonal antibodies. Monoclonal antibodies can be made by fusing a myeloma cell with an antibody-producing lymphocyte. Such hybridomas produce virtually unlimited quantities of monoclonal antibodies *in vitro*. Monoclonal antibodies excel in the identification of antigens because cross-reacting antibodies are absent thus they are highly specific.

There are two basic methods for identification of unknown organisms by using sera containing various known antibodies; one is agglutination for particulate antigens and another is precipitation for soluble antigens. Other immunoassay methods include, fluorescent antibody technique (FAT), enzyme-linked immunosorbent assay (ELISA) and Western blot analysis.

Agglutination

Agglutination reactions are among the most easily performed of immunological tests. Their usefulness in presumptive identification of bacteria has long been recognized and for bacterial fish pathogens has been reconfirmed and documented. With as few as a dozen antisera, it is possible to confirm the identity of the majority of known bacterial fish pathogens. Agglutination has provided valuable information on the serological relation of bacterial fish pathogens, including species within genera and strains of the same species.

In this reaction, the antigen is particulate (e.g., bacteria and red blood cells) or is an inert particle (latex beads) coated with an antigen. Because it is divalent or multivalent, the antibody cross-links the antigenically multivalent particles and forms a latticework, and clumping (agglutination) can be seen (Figure 10-1). The procedure is used both to demonstrate the presence of antibodies in serum and to identify antigens on microbial cell surfaces. The principle is the same in both applications. If the serum contains antibodies against a surface antigen, they will agglutinate the bacterial cells. Using a variety of typing sera,

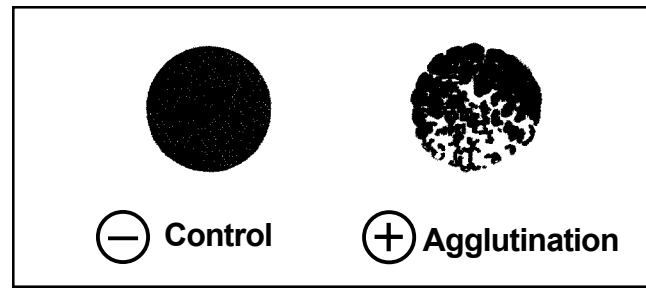

Figure 10-1. Slide agglutination. Negative (-) and positive (+) reactions

bacteria can be identified and classified. If a constant amount of a bacterial cell suspension is mixed with graded volumes of dilutions of homologous antiserum, one obtains a measurement of the concentration of antibodies in the serum. The term used to describe the concentration of antibody in serum is "titer", which is the reciprocal of the highest dilution producing a definite reaction.

For use in agglutination tests, bacteria harvested from broth or agar medium may be resuspended adequately. Sometimes bacteria have a tendency to aggregate spontaneously, or "autoagglutinate", making their use in agglutination tests unfeasible. If spontaneous agglutination is observed with a bacterial suspension to be used as a test organism, there are several modifications that can be used to eliminate this reaction. If the preparation was formalinized before removal from the growth medium, sometimes washing the bacteria in neutral buffered saline before formalinization reduces non-specific agglutination. If agglutination still occurs, resuspension of the organisms in a 0.1% protein solution such as non-fat dry milk to block sites of adherence may be a remedy. Further autoagglutination may necessitate heating of bacterial suspension by immersion in a boiling water bath for 1 to 10 minutes. If none of these procedures eliminate the autoagglutination problem, the particular organism may not be amenable to this procedure.

Agglutination test has been widely used in detecting bacterial fish pathogens belonging to the genera *Vibrio*, *Pasteurella*, *Aeromonas*, *Yersinia*, *Edwardsiella* and *Pseudomonas* (Toranzo et al., 1987).

Precipitation

In this reaction, the . The antibody cross-links antigen molecules in variable proportions, and aggregates (precipitates) form. In the zone of equivalence, optimal proportions of antigen and antibody combine; the maximal precipitates forms, and the supernatant contains neither an excess of antibody nor an excess of antigen. In the zone of antibody excess, there is too much antibody for efficient lattice formation, and precipitation is less than maximal. In the zone of antigen excess, all antibody has combined but precipitation is reduced because many antigen-antibody complexes are too small to precipitate.

Procedures involving soluble antigens have been used to study the antigenic composition of fish pathogens (bacterial, viral and parasite) and to a lesser extent a diagnostic tools.

Precipitin reactions can be done in semisolid medium (agar):

1. Single diffusion

Using this method, it is possible to quantitate the concentration of anti body and antigen in a solution. The antigen diffuses in a radial direction out from the well and a precipitin ring develops when the reactants are close to their optimal proportions (Figure 10-2). At the equivalence point, when the ring is stationary, the square of the diameter or area of the ring is directly proportional to antigen concentration. Conversely, though less sensitive, antigen can be mixed into the agar and the amount of antibody in a sample can be determined. Consequently, it is possible to calibrate the plate using a pre-determined constant amount of antibody (or antigen) in the agar and placing known concentrations of antigen (or antibody), or sample dilutions in the wells. By calibrating the method, such radial immunodiffusion is used to measure IgG, IgM, complement components, and other substances in the serum.

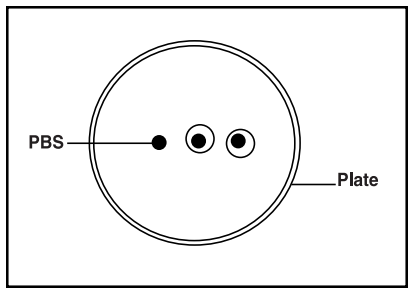

Figure 10-2. A representation of a single radial immunodiffusion measurement. Typical rings obtained with positive rabbit serum and no ring with phosphate buffered saline (PBS) as negative control

In some cases, poor resolution occurs with complex antigenic mixtures although the method is relatively sensitive when soluble antigens are employed. Furthermore, the length of time for visualization of results depends on antigenic type and/or molecular mass. This technique is best used with low molecular weight antigens and is unaffected by molecular charge. With high molecular weight antigens, the diffusion time is longer for formation of the precipitin ring maximum diameter. The use of tannic acid as a precipitin intensifying agent increases the plate sensitivity and allows detection of lower antigen levels in serum. The method offers a limited benefit and is insensitive for diagnostic purposes. It requires higher antigen concentrations in the test mixture since low amounts may result in lack of precipitation of the samples used.

The method has been used infrequently in fish immunological studies and then only for estimation of the immunoglobulin concentrations in both normal and immune serum. The concentration of immunoglobulins produced in response to injection with *Salmonella* bacterial and/or red blood cell (RBC) antigens have been measured in catfish (Ourth, 1986). Serum IgM levels have been measured in rainbow trout naturally infected with VHS virus and ERM bacteria (Olesen and Jorgensen, 1986).

2. Double diffusion

In the gel diffusion technique, gels, usually clarified agar are used as matrices for combining diffusion with precipitation. Antigen and antibody are placed in different wells in agar and allowed to diffuse towards each other (passive diffusion) and precipitation results where the optimal antibody/antigen ratios have been reached (Figure 10-3). This method (Ouchterlony) can be used either to detect the number of major components in an antigenic mixture or identify the presence of homologous and heterologous molecules in an antigenic extract based on the specific recognition capacity of a prepared antiserum.

If two different samples contain identical or related antigen epitopes, the precipitin lines are confluent and meet to give a single arc, thus a denoting a

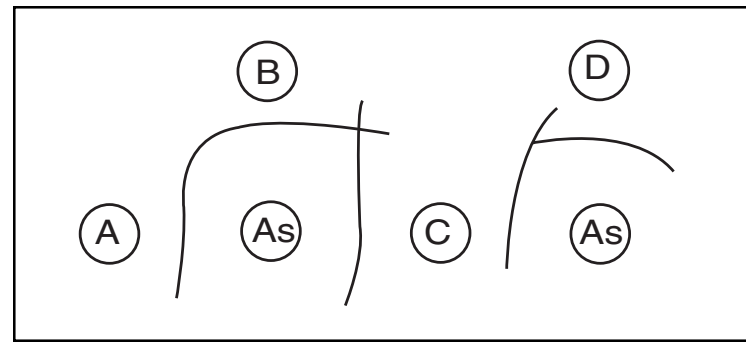

Figure 10-3. Double diffusion precipitation reactions (Ouchterlony) in gel. As – antiserum in wells; A, B, C, D – antigens in wells; A and B – reaction of identity; B and C reaction of nonidentity; C and D – reaction of partial identity (cross-reaction; the "spur" is caused by the fraction of antibody that was not precipitated by antigen D)

reaction of identity. If antigens in the two samples are dissimilar or unrelated and possess no common epitopes then the precipitin lines are independent of each other and cross over, indicating a reaction of non-identity. When the antigen samples contain two molecular species, a reaction of identity occurs when epitopes are similar to both of the molecular types but an additional epitope on one of the antigen species would give rise to a line of non-identity such that a spur is formed. This is also referred to as a reaction of partial identity since the antisera would detect the unique component on one but not the other antigen of similarity. Lastly, if the two molecular species have one epitope in common and each has an epitope(s) not present in the other, partial identity occurs and two spurs are formed.

The method has been employed frequently to determine the serological relationships between fish bacterial strains on the basis of lipopolysaccharide types, between *Vibrio* bacteria isolated from other marine teleosts (Johnsen, 1977), in the identification of extracellular *Vibrio* toxins pathogenic to eels and ayu (Kanemori et al., 1987) and for comparison of antigenicity of *Edwardsiella tarda* after injection into ells (Salati and Kusuda, 1985). It was also used to diagnose BKD (Kimura et al., 1978) and to determine the serological differences of *Photobacterium damsela* subsp. *piscicida* isolates (Kawahara et al., 1998).

3. Immunoelectrophoresis

This technique is a widely used method in fish immunology and combines electrophoresis with immunoprecipitation. It has a much better resolution than gel diffusion. In this system, the components of the antigen will first be separated by electrophoresis. Separation of the components occurs due

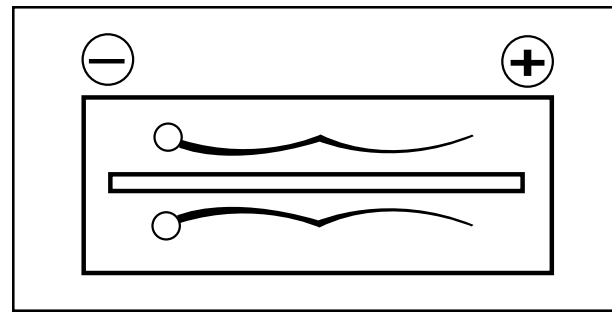

Figure 10.4. Immunoelectrophoretic pattern of *Vibrio harveyi* LPS. In the well above the trough, LPS in the supernatant after ultracentrifugation of the water layer while in the well below the trough, purified LPS. In the trough, anti-*V. harveyi* serum

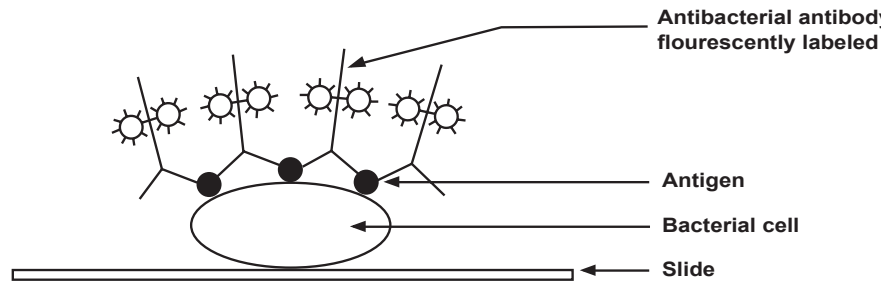

Figure 10-5. Direct fluorescent antibody technique (DFAT)

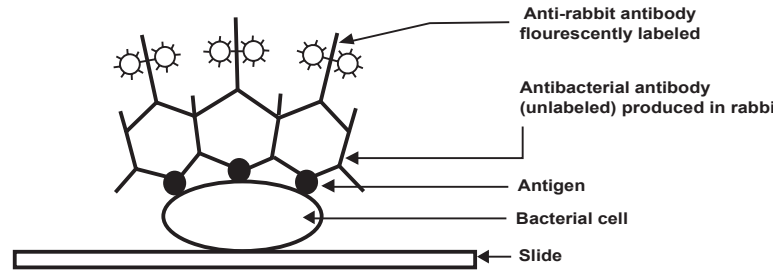

Figure 10-6. Indirect fluorescent antibody technique (IFAT)

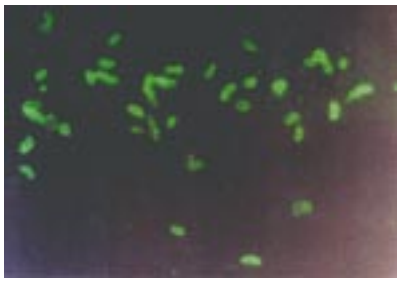

Figure 10-7. Indirect fluorescent antibody technique (IFAT) staining of an impression smear prepared from *Vibrio penaeicida* cell suspension

to different electrophoretic mobilities caused by charges on the molecules. After the antigen has been separated into its components, antiserum will be put into a channel cut parallel to the direction of the electrophoresis. From this channel, the antibodies will diffuse torwards the electrophoretically separated antigen components, and vice versa. As the antigen and antibody diffuse toward each other, they form a series of arcs of precipitate (Figure 10-4). This permits the serum proteins to be characterized in terms of their presence, absence, or unusual pattern. The separated components are then visualized on the plate by precipitin band formation due to the diffusion of a specific antiserum into the agar parallel to the current direction. With this method, the antigenic complexities of various sera can be determined, detect the purity of one component system or isolated fractions, detect and to determine the number of components in a multicomponent system and to demonstrate antibody heterogeneity.

The test was used to identify IPN virus in cell culture (Dea and Elazhary, 1983) and determine the serological characteristics of atypical strains of *Edwardsiella tarda* isolated from sea breams (Costa et al., 1998).

Fluorescent Antibody Technique (FAT)

Searching for bacteria in fish tissue is easier than defining viral infections by FAT. In most cases when antisera against viral fish pathogens are used, the fish tissues suspected of being infected are processed, filtered and placed in culture on fish cell lines; the purpose being to increase the amount of virus.

The FAT makes use of the feature that some dyes (fluorescein isothiocyanate, rhodamine isothiocyanate, Texas red) fluoresce when viewed under ultraviolet

(UV) light. These dyes can be used as tags, conjugated to target antibody molecules, and when the antibody complexes with the specific antigen, the complex is "lighted". A major advantage of being able to visualize the antigen-antibody reaction on the pathogen gives the pathologist confidence in diagnosis. This technique generally detects surface-associated antigens. Slide formats, with organisms fixed directly to the slides, are used for bacteria. After fixation, antigen is incubated with specific antibody. This may be labeled directly with fluorescein to allow visualization of antibody (direct FAT) (Figure 10-5) or, alternatively, a fluorescein-labeled antibody conjugate is added in a second stage to detect bound antibody (indirect FAT) (Figures 10-6 and 10-7). Slides are then examined with an ultraviolet microscope, and the intensity of fluorescence, relative to appropriate controls, is scored in an arbitrary scale from -, +, to + + + + +. Typical assays can be performed in 2-3 h.

Immunofluorescence is widely used for the detection of antigen or antibody in fish, typing and identification of a range of microorganisms. FAT has been used to detect antibodies to *Aeromonas liquifaciens* in fish (Lewis and Savage, 1972). Rapid FAT diagnosis has been developed to detect Pseudotuberculosis in yellowtail (Kitao and Kimura, 1974), *Renibacterium salmoninarum* in salmo-nids (Bullock et al., 1980) and *Vibrio penaeicida* in kuruma prawn (de la Peña et al., 1992). FAT has also been developed for rapid diagnosis of infectious hematopoeitic necrosis virus (IHNV) (LaPatra et al., 1989) and red sea bream iridovirus (Nakajima et al., 1995) in fish.

Enzyme-Linked Immunosorbent Assay (ELISA)

The covalent attachment of enzymes to antibody molecules creates an immunological tool possessing both high specificity and sensitivity. The technique makes use of antibodies to which enzymes have been covalently bound such that the enzyme's catalytic properties and the antibody's specificity are unaltered. Typical linked enzymes include peroxidase, alkaline phosphatase and b-galactosidase, all of which catalyze reactions whose products are colored and can be measured in very low amounts.

The ELISA is one of the most powerful of all immunochemical techniques. It employs a wide range of methods to detect and quantitate antigens or antibodies and to study the structure of antigens. There are many variations on the ways the immunoassays can be performed. Immunoassays are classified on

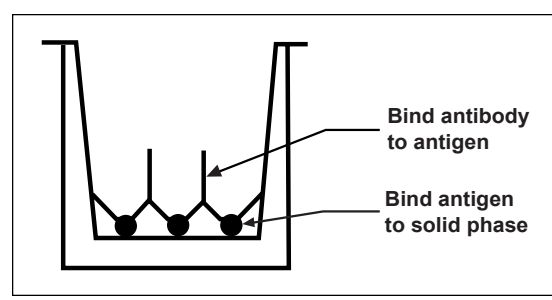

Figure 10-8. Antibody-capture assay

the basis of methodology and within each group, the principle and the order of the steps are similar. For example, by changing certain key conditions, an as-say can be altered to determine either antigen or antibody level. Although the steps are similar, the assays yield different results.

The three classes of ELISA tests are:

1) antibody capture assays,
2) antigen capture assays and
3) two-antibody sandwich assays

Antibody capture assay can be used to detect and quantitate antigens or antibodies and compare the epitopes recognized by different antibodies. The general protocol; an unlabeled antigen is immobilized on a solid phase and the antibody is allowed to bind to the immobilized antigen (Figure 10-8). The antibody can be labeled directly or can be detected by using a labeled secondary reagent like goat anti-rabbit or anti-mouse IgG antibody containing conjugated enzyme that will specifically recognize the antibody. Following the addition of enzyme substrate, a color is formed, and the amount of antibody relative to the specific antigen is quantitated from the intensity of the color reaction measured by an ELISA plate reader, a modified spectrophotometer The color formed is proportional to the amount of antibody that is bound. The three factors that will affect the sensitivity of a labeled antibody assay are (1) the amount of antigen that is bound to the solid phase, (2) the avidity of the antibody for the antigen and (3) the type and number of labeled moieties used to label the antibody. Variations in methodology under antibody capture assay are (1) detecting and quantitating antibodies using antigen excess assays, (2) comparing antibody binding sites using an antibody competition assay, (3) detecting and quantitating antigens using antibody excess assays and (4) detecting and quantitating antigens using antigen competition assays.

Antigen capture assays are used primarily to detect and quantitate antigens (Figure 10-9). The amount of antigen in the test solution is determined using a competition between labeled and unlabeled antigen. Unlabeled antibody is bound to the solid phase either directly or through an intermediate protein, such as an anti-immunoglobulin antibody. The antigen is purified and labeled. A sample of the labeled antigen is mixed with the test solution containing an unknown amount of antigen and the mixture is added to the bound antibody.

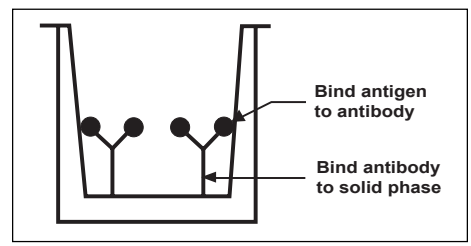

Figure 10-9. Antigen-capture assay

The antigen in the test solution will compete with the labeled antigen for binding to the antibody-matrix. Unbound proteins are removed by washing, and the amount of labeled antigen bound to the matrix is measured. If the un-

known solution contains a high concentration of antigen, it will compete effectively with the labeled antigen and little or none of the labeled antigen will bind to the antibody. Following a wash, the enzyme activity of the bound material in each microtiter well is determined by adding the substrate of the enzyme. Color development in individual wells of the plate is assessed by eye or more commonly, quantified with a commercially available ELISA plate reader-a modified spectrophotometer The color formed is proportional to the amount of antigen originally present. It is necessary to determine cut-off values to discriminate between true positive reactions and background reactivity by including appropriate positive and negative controls with each series of assays. The sensitivity of the labeled antigen assay will depend on three factors: (1) the number of antibodies that are bound to the solid phase, (2) the avidity of the antibody for the antigen and (3) the specific activity of the labeled antigen.

Two-antibody sandwich assays are used primarily to determine the antigen concentration in unknown samples. The assay requires two antibodies that bind to non-overlapping epitopes on the antigen (Figure 10-10). Either two monoclonal antibodies that recognize discrete sites or one batch of affinity-purified polyclonal antibodies can be used. To use the assay, one antibody is

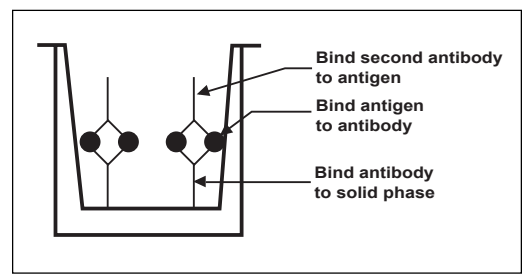

Figure 10-10. Two-antibody sandwich assay

purified and bound to a solid phase and the antigen in a test solution is allowed to bind. Unbound proteins are removed by washing and the labeled second antibody is allowed to bind to the antigen. The conjugate comprises antibody chemically linked to the enzyme horseradish peroxidase. After a further incubation, excess conjugate is removed by washing the wells and the bound peroxidase is determined by adding chromogen in substrate buffer. Color development (blue) is proportional to the original bacterial content in the sample and can easily be read by the naked eye. The assay can also be amplified at this stage thus increasing sensitivity by adding stop solution. The color obtained changes from blue to yellow and results are read using an ELISA plate reader with a 450 nm filter. The inclusion of appropriate positive and negative controls are essential. In addition, during the optimization of the test, check for endogenous peroxidase activity in the tissue samples being used. If activity is found, then this may be overcome by altering the method of extraction (e.g. heating step). It may be necessary to change to a different enzyme system (e.g. alkaline phosphatase) if activity is very high (e.g. spleen tissue). The major advantages of this method are that the antigen does not need to be purified prior to use and that the assays are very specific. The major disadvantage is that not all antibodies can be used. The sensitivity of the assay is dependent on four factors: (1) the number of molecules of the first antibody that are

bound to the solid phase, (2) the avidity of the first antibody for the antigen,(3) the avidity of the second antibody for the antigen and (4) the specific activ-ity of the labeled second antibody.

Two types of detection systems are commonly used for ELISA: iodinated re-agents and enzyme-labeled reagents. Assays that use iodinated reagents are easier to quantitate while enzyme assays will often yield a quicker result. Ei-ther iodinated or enzyme-labeled reagents can be used for direct or indirect methods. When using direct detection methods, the antibody or antigen is purified and labeled while for indirect detection, a labeled secondary reagent that will bind specifically to an antibody is used. A third variation that uses properties of both direct and indirect detection is the biotin-streptavidin sys-tem. Here, the antigen or antibody is purified and labeled with biotin. The biotinylated reagent is detected by binding with streptavidin that has been la-beled with iodine or an enzyme.

ELISA test has been used to detect *Aeromonas salmonicida* in fish tissue (Adams, 1990), clinical cases of enteric red mouth and furunculosis in fish farms (Austin et al., 1986), *Vibrio parahaemolyticus* (Adams, 1991) and *V. harveyi* (Song et al., 1992) in penaeid shrimp. ELISA has also been developed for rapid detection of viral haemorrhagic septicaemia virus (Olesen and Jorgensen, 1991) and striped jack nervous necrosis virus (Arimoto et al., 1992) in fish.

Western Blotting

Western blotting is a rapid and sensitive assay for the detection and characterization of proteins. The technique allows one to identify particular proteins by utilizing the specificity inherent in antigen-antibody recognition. This technique is powerful, since it combines electrophoretic separation of proteins, glycoproteins and lipopolysaccharides with immunological identification. Once such antigens have been detected, they can be further characterized by Western blotting. Both techniques can utilize either polyclonal or monoclonal antibodies.

Initially, a sample is subjected to electrophoresis to separate antigens according to their charge and size, or size alone. A second electrophoretic step transfers the antigens from the gel to an immobilizing surface, such as nitrocellulose

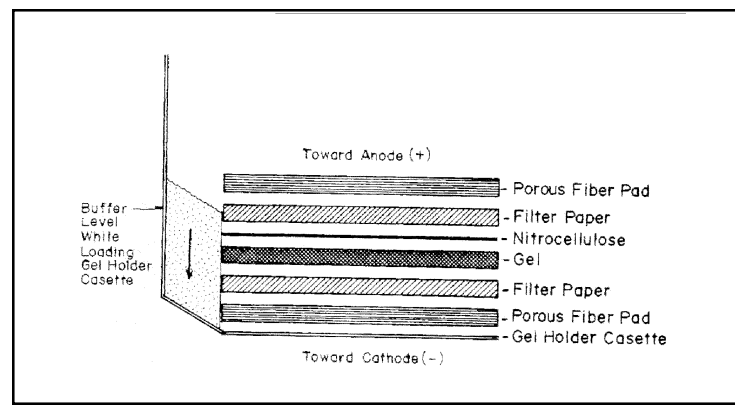

Figure 10-11. Western blotting. Organization of materials inside the gel cassette holder for transfer of proteins from the gel to nitrocellulose paper

paper where they are bound irreversibly (Figure 10-11). After this transfer, the paper is blocked with 3% gelatin in PBS to prevent nonspecific binding of anti-body and probed with a specific enzyme-conjugated antibody (horseradish peroxidase-anti-immunoglobulin conjugate). A chromogenic substrate is then added to determine which electrophoretic band is bound by the antibody.

The technique is useful for a number of purposes including characterization of unknown antigens or antibody specificities, confirmation of the presence of bacterial antigens in sera or tissues and detection of seropositive individuals which have been exposed to a pathogen. The primary advantage of Western blotting, as opposed to other immunoassays, is the high degree of specificity in resolving distinct antigens. However, there are two disadvantages: first, it is mainly a qualitative assay and quantification of antibody or antigen is difficult; and second, if the antigen sample must be denatured (such as in SDS-PAGE), antigenic activity may be reduced or destroyed. An initial consideration for successful Western blotting is the empirical determination of the optimal per-centage of acrylamide for resolution of the antigens of interest. Next, it must be determined whether the antigens are capable of binding to the nitrocellu-lose. Finally, the antigen detection procedure must be highly specific and sen-sitive.

The technique can be used to detect as little as 1 ng of a protein antigen that has been previously separated under denaturing conditions by SDS-PAGE, provided that an antibody that recognizes the denatured form of the protein is available. The entire procedure can be completed in 1 to 2 days, depending on transfer time and type of gel.

Western blotting has been useful for characterizing the specificities of polyclonal antisera (rabbit and salmonid) and monoclonal antibodies to extracellular and cell surface antigens of *Renibacterium salmoninarum* (Wiens and Kaattari, 1989). It has also been used in the detection of yellowhead virus and white spot syndrome virus in penaeid shrimp (Nadala et al., 1997; Magbanua et al., 2000).

MOLECULAR BIOLOGY TECHNIQUES

The central foci of molecular biology are the nucleic acids, deoxyribonucleic acid (DNA) and the ribonucleic acid (RNA). Nucleic acids encode the genetic information specifying the primary structure of all proteins unique to an organism. Together with lipids and extracellular supporting stroma, they create cellular activity and physiological function. Thus, biological functions can be understood in part by examining the interrelationships between these key components. RNA and DNA are composed of four separate building blocks called nucleotides. Each of the four nucleotides contains a nucleic acid base (A, adenine; G, guanine; T, thymine; C, cytosine), a deoxyribose sugar moiety and a phosphoester for DNA. For RNA, the same bases as in DNA are present, except that uridine (U) is substituted for T and a ribose moiety is present instead of the deoxyribose. The nucleotides are connected one to another to form a chain-like arrangement, which comprises the nucleic acid's sequence. RNA is

composed of a single strand whereas DNA is composed of two paired strands. In order for the paired strands to match up, they must face each other in the opposite or complementary direction. The complementary strands of DNA are kept together primarily by the hydrogen bonds that form between the bases A and T (2 bonds) as well as C and G (3 bonds). It is this hydrogen bonding between the matched base pairs A and T (or, for RNA, A and U) as well as C and G that is the foundation of all molecular biological tests. Although one given base pair match of AT or GC would separate easily, there is strength in numbers and the more base pair matching, the greater the number of hydrogen bonds between the two DNA strands and the less likely they are to separate.

In the hybridization of nucleic acid strands, when two DNA strands meet, they orient each other in opposite or antiparallel directions to allow base pair matching to occur. If no base pair matching is present, they go their separate ways. However, if there is sufficient base pair matching they will join together of hybridize. The specific term used to describe the degree of base pair match-ing that determines if the strands stay together is homology. How much ho-mology is needed for two strands to stay together? Although it is true "the more, the better," another important variable is how close the base pair matches are to one another. Adjacent base pair matches in a sequence will hold together more strongly than the same number of base pair matches dis-persed over the DNA sequence. Dispersed base pair matches are typical unre-lated DNA strands whereas clustered base pair matches are expected for re-lated complementary DNA molecules. Clearly, if two DNA strands are com-pletely homologous and have 100% base pair matching then the strands would tend to remain hybridized under most conditions. Conversely, hybridized strands with poor homology (e.g., only 10% of base pairs matched) would tend to dissociate or denature readily under most conditions. Whether hybridized strands with intermediate homology - where, for example, 50% of the base pairs matched - would remain hybridized would depend greatly on the reaction conditions.

Given that hydrogen bonds are the glue that keep two hybridized strands to-gether and that many chemicals and conditions can affect hydrogen bonding, a term is needed that describes whether the hybridization reaction conditions relatively favor or disfavor hydrogen bonding - stringency. Under low strin-gency conditions, hybridized strands with intermediate homology would tend to remain hybridized whereas hybridized strands with poor homology would dissociate. At high stringency conditions, only hybridized strands with strong homology would tend to remain hybridized.

Another key term, the melting temperature, or commonly abbreviated, T_m. If one takes two strands of DNA that share homology and hybridizes them, at any given time some of the strands will remain hybridized whereas others will have separated. The ratio of hybridized/denatured DNA strands in the reaction will vary depending on the degree of homology as well as any condition that may affect hydrogen bonding between matched base pairs such as formamide con-centration and temperature. The melting temperature is defined as that tem-perature under the specific reaction conditions where one half of the hybrid-ized strands are still hybridized and the other half are denatured.

Two molecular biology-based techniques discussed here are: gene probe and polymerase chain reaction (PCR).

Gene probe assays

The power of DNA diagnostics is a consequence of two facts: (1) nucleic acids can be rapidly and sensitively measured, and (2) the sequence of nucleotides in a given DNA molecule is so specific that hybridization analyses can be used for reliable clinical diagnoses.

One of the most powerful analytical tools available is nucleic acid hybridization. Instead of detecting a whole organism or its products, hybridization detects the presence or absence of specific DNA sequences associated with a specific organism. To identify a microorganism through DNA analysis, you must have available nucleic acid probe to that microorganism, a single strand of DNA containing sequences unique to the organism. The unlabeled strand in the sample being analyzed that is homologous to the probe is called the target. If a microorganism in a specimen contains DNA sequences complementary to the probe, the two sequences can hybridize forming a double stranded molecule. To detect that a reaction has occurred, the probe is labeled with a **re**porter molecule, either a radioisotope, an enzyme, or a fluorescent compound that can be measured in small amounts following hybridization. Depending on the reporter used (radioisotopes are the most sensitive), as little as 0.25 mg of DNA per sample can be detected.

Several techniques have evolved based on the ability of a labeled probe to bind to and thus permit the detection of the target nucleic acid sequence of interest. One approach is to extract the DNA, both target and non-target, from a sample and bind it to a filter where it can be hybridized with the labeled probe. This is called filter hybridization. Often times the sample DNA is directly placed on the filter with the aid of a vacuum manifold, which has slot-like spaces for each sample, hence the term slot blot or dot blot hybridization. Dot blotting is rapid, simple technique for the quantification of RNA or DNA target sequences without prior electrophoretic separation. This method differs only in the shape of the immobilized nucleic acid spot deposited on the membrane. Nucleic acid is applied to a dry nitrocellulose filter and allow to dry. The resulting “dots” are variable in size, making accurate estimates of target sequence concentration difficult. Alternatively, the sample DNA may first be separated according to size and configuration by electrophoresis on a gel and then transferred to a filter - this is termed Southern blot hybridization. As can DNA fragments, RNA molecules can be separated on the basis of size by gel electrophoresis and can be immobilized on membranes by a process referred to as Northern blot hybridization. Detection of target sequences in Southern, Northern and dot blots is carried out under essentially identical conditions. Three basic processes are involved: (1) prehybridization, which saturates nonspecific DNA binding sites on the membrane with random DNA and polymers; (2) hybridization, during which specific labeled probes are annealed to target sequences; and (3) washing, to remove unhybridized and imprecisely hybridized probe. In either of these techniques, the tissue must be destroyed thus precluding direct histological correlation.

In the other major strategy based on hybridization of a target and a probe, the target DNA is not extracted but rather kept in the intact cell where it may bind to the probe - this is the *in situ* hybridization. The principal advantage of *in situ* over other molecular techniques is it provides information about the location of the target nucleic acids within cells and/or tissues. Pathologists are able to look for specific nucleic acids and to study the cellular and tissue morphology of the sample. A misconception about *in situ* hybridization is that one needs to use radiolabeled probes in order to maximize its sensitivity however, recent and dramatic advances in nonisotopic labeling and, more importantly, detection systems has greatly enhanced the sensitivity using such common labels as biotin and digoxigenin. The most common problem encountered with the use of nonradioactive systems is background. Background may be defined as the presence of a hybridization signal with a specific probe in areas of the tissue where the signal should not be present. Background is often the result of nonspecific binding of the probe to nontarget molecules. Two ways to deal with background are to decrease the concentration of the probe and/or to increase the stringency of the post-hybridization wash. Another common problem is poor tissue morphology due to overtreatment with the protease solution. Decreasing the time of digestion or the concentration of the protease tenfold will solve this problem. Another is occasionally tissue sections may fall off. The problem rests with incorrect silanization of the slides. The most obvious potential problem is the absence of a hybridization signal. In dealing with a negative signal, you have to check the proper fixative (no heavy metals or picric acid), alkaline phosphatase conjugate, chromagen, denaturing temperature (=95°C) and the tissue was deparaffinized. The target molecules are nucleic acids. Nucleic acids are complexed with proteins in the cell; when a tissue is embedded in a complex matrix, the nucleic acids are cross-linked to that matrix. Thus, major challenges of *in situ* hybridization are to make target nucleic acid available to the probe; and once proper hybrids are formed, to stabilized them without destroying the cell morphology.

An important difference between filter and *in situ* hybridization is the detection threshold. Detection of a DNA sequence by *in situ* hybridization implies a selective increase in its numbers due to, for example, oncogene amplification or viral proliferation. On the other hand, only one virus need to be present per every 100 cells for the Southern blot test to detect it, though the *in situ* test would be scored as negative. *In situ* is relatively insensitive test and is usually negative in situations such as occult or latent infection by a virus where the number of target DNA sequences per cell is low. Why is there such a disparity in detection threshold? The primary reason is the probe may find it more difficult to find the target if it has to traverse the labyrinth of nuclear proteins and nucleic acids in *in situ* analysis relative to more "naked" DNA that has been attached to a filter in dot blot or Southern blot hybridization. Second, the extraction and purification of DNA characteristic of filter hybridization leads to a concentrating effect of rare nucleic acid sequences.

Nucleic acid probes offer many advantages over immunological assays. Nucleic acids are much more stable than proteins to high temperatures, high pH, organic solvents and other chemicals. In addition, nucleic acid probes are more defined entities than antibodies.

Gene probes are extensively used as diagnostic tools to detect white spot syndrome virus (WSSV) (Chang et al., 1996; Chang et al., 1998), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Mari et al., 1993) and hepatopancreatic parvovirus (HPV) (Mari et al., 1995) of penaeid shrimp and viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV) in fish (Ristow et al., 1991).

Selected features of three major hybridization assays

	In situ	Southern blot	Dot blot
Detection threshold	20 copies/cell	1 copy/100 cells	1 copy/200 cells
Samples	fresh or fixed	fresh	fresh
Background	low	low	low-high[a]
Time	≦ 1 day	2-7 days	1-5 days
Cell localization	yes	no	no
Equipments	none	transfer unit gel cast/electro-phoresis unit	vacuum manifold
Samples/run	≤ 25	≤ 15	≤ 75
Detection of latent infection	no	yes	yes

[a]Depending on stringency.

Polymerase Chain Reaction (PCR)

Various molecular biology techniques continue becoming more important in fish and srimp farming, particularly in detection and prevention of various diseases. One of the most prominent techniques is the PCR. No technique has had a greater impact on the practice of molecular biology than the PCR. The PCR for amplification of specific nucleic acid sequences was introduced by Saiki et al. (1985) and has subsequently proved to be one of the most important scientific innovations of the past decade. With this technique, one can rapidly detect a virus or bacteria, few copies of mRNA, rapidly synthesize, clone and sequence virtually any segment of DNA. Despite the incredible power of the technique there has been one major limitation that is the DNA must be extracted from the sample one can not correlate PCR results with the pathological features of material being tested.

The development of PCR means that small amounts of DNA no longer limit molecular biology research or DNA-based diagnostic procedures. The technique is continuously improving and its full impact on molecular diagnostics is yet to come. PCR currently has many applications, including analysis of ancient DNA from fossils, amplification of small DNA amounts for analysis by DNA fingerprinting, mapping the human genome and also those of other species, and detection of microorganisms present in low densities in water, food, soil or other organisms. In aquaculture, PCR is a valuable tool for the preven-

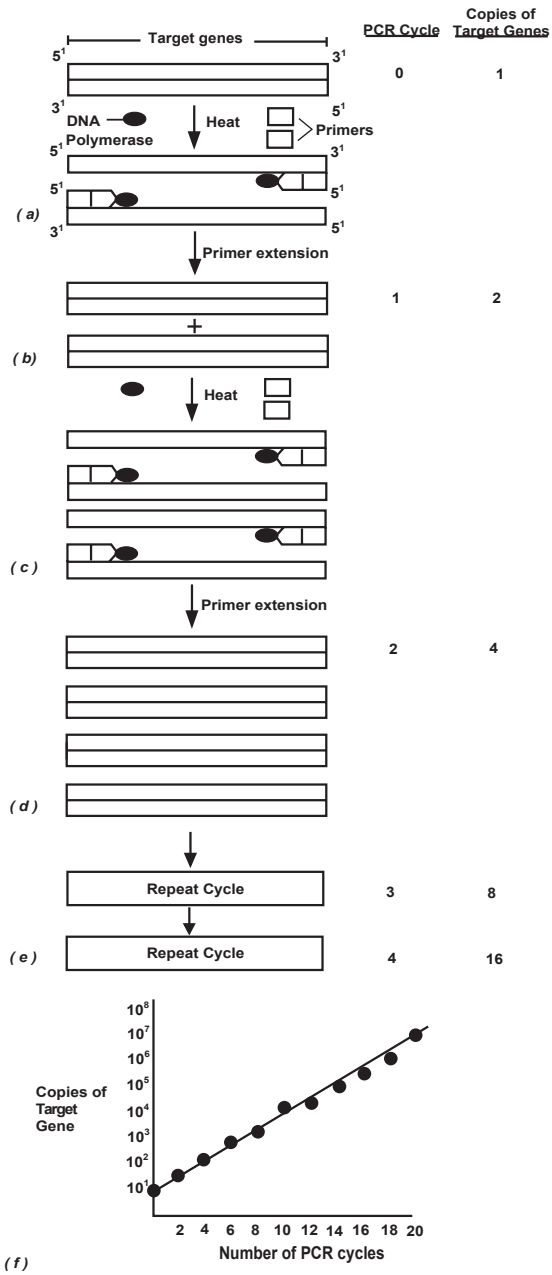

Figure 10-12. Polymerase chain reaction. (a) Target DNA is heated to separate the strands and two primers, one complementary to the target strand and one to the complementary strand, are added along with DNA polymerase. (b) Following reannealing, primer extension yields a copy of the original double-stranded DNA. (c) Further heating, primer addition, and primer extension yields a second double-stranded DNA. (d) The second double-stranded DNA. (e) Two additional PCR cycles yield 8 and 16 copies, respectively, of the original DNA sequence. (f) Effect of running 20 PCR cycles on a DNA preparation originally containing 10 copies of a target gene

tion, control and management of various diseases. For fish and shrimp farmers, it permits fast, widespread, and sensitive screening of virus carriers, and also for early or light infections. The tests can be carried out non-destructively by using body fragments, blood or feces from fish and shrimp tested. PCR can be used to screen both broodstock animals and also larvae before stocking. PCR is rapidly becoming a critical instrument to detect fish and shrimp pathogens.

PCR assay has been widely used in the detection of fish viruses like stripe jack nervous necrosis virus (SJNNV) (Nishizawa et al., 1994), red sea bream iridovirus (RSIV) (Kurita et al., 1998), aquatic birnaviruses (Williams et al., 1999) and shrimp viruses like white spot syndrome virus (WSSV), monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvo virus (HPV), baculovirus penaei (BP), Taura syndrome virus (TSV), yellow head virus (YHV) and baculoviral midgut gland necrosis virus (BMN) (Lightner, 1996; Wongteerasupaya et al., 1997; Lightner and Redman, 1998; Tapay et al., 1999; Hsu et al., 2000; Magbanua et al., 2000). The assay has also been used in the detection of *Vibrio penaeicida* in shrimp (Genmoto et al., 1996; Nakai et al., 1997) *Aeromonas salmonicida* subspecies *salmonicida* (Miyata et al., 1996), *Pasteurella piscicida* (Aoki et al., 1997) and *Lactococcus garvieae* (Aoki et al., 2000) in fish.

PCR uses a thermostable polymerase to produce multiple copies of specific nucleic acid region quickly and exponentially. For example, starting with a single copy of a 1 kb DNA sequence, 10^{11} copies (or 100 ng) of the same sequence can be produced within a few hours. Once the reaction has occurred, a number of methods for identification and characterization of the amplification products are then applicable, of which the simplest is to identify the products according to their size following migration in the agarose gels. For many diagnostic applications, the simple visualization of a PCR product of characteristic size is a significant outcome since it indicates the presence of the target DNA sequence in the original sample.

Basic principle

Each PCR amplification is subdivided into three steps which are repeated in cycles as indicated below (Figure 10-12):

(1) **melting or denaturation** (strand separation) of the double strand DNA (one to several minutes at 94-96°C);

(2) **annealing** of the two primers to opposite DNA strands (one to several minutes at 50-65°C); and

(3) **extension** of the primers by polymerase-mediated nucleotide additions to produce two copies of the original sequence (one to several minutes at 72°C).

During cellular DNA replication, enzymes first unwind and denature the DNA double helix into single strands. After the DNA is denatured, one more event must occur before DNA synthesis may be catalyzed by the DNA polymerase. It must find an area of transition from single stranded to double stranded DNA. At proper temperature typically around 55°C, sufficient primer-target DNA hybridization occurs which leads to the synthesis of complementary strands essential for the amplification step. During PCR, high temperature is used to separate the DNA molecules into single strands, and synthetic sequences of single-stranded DNA (20-30 nucleotides) serve as primers. Two different primer sequences are used to bracket the target region to be amplified. One primer is complementary to one DNA strand at the beginning of the target region; a second primer is complementary to a sequence on the opposite DNA strand at the end of the target region.

Components of a typical PCR	
Tris-HCl (pH 8.3)	20 mM
$MgCl_2$	2.5 mM
KCl	25 mM
dNTPs	50 mM each
Primer 1 Primer	20 pmol
2	20 pmol
Taq polymerase	2.5 units
Template DNA	10-100 ng
Mineral oil	optional

As amplification proceeds, the DNA sequence between the primers doubles after each cycle. Following thirty cycles, a theoritical amplification factor of one billion is attained.assuming **100% efficiency** during each cycle. The final number of copies of the target sequence is expressed by the formula, $(2^n - 2n)x$, where:

n - number of cycles;
$2n$ - first product obtained after cycle 1 and second products

obtained after cycle 2 with undefined length;
x - number of copies of the original template.

There are factors that act against the process being 100% efficient at each cycle. Their effect is more pronounced in the later cycles of PCR. Normally, the amount of enzyme becomes limiting after 25-30 cycles, which corresponds to about 10^6-fold amplification, due to molar target excess. The enzyme activity also becomes limiting due to thermal denaturation of the enzyme during the process. Another factor is the reannealing of target strands as their concentration increases. The reannealing of target strands then competes with primer annealing.

Two important innovations were responsible for automating PCR. First, a heat-stable DNA polymerase was isolated from the bacterium *Thermus aquaticus* that lives in hot springs. Hence, the term *Taq* (DNA) polymerase came to be. This enzyme remains active despite repeated heating during many cycles of amplification. Second, thermal cyclers were invented which automatically control the repetitive temperature changes required for PCR.

Following amplification, the PCR products are usually loaded into wells of an agarose gel and electrophoresed. Since PCR amplifications can generate microgram quantities of product, amplified fragments can be visualized easily following staining with a chemical stain such as ethidium bromide. The important point to remember is that the amplification is selective - only the DNA sequence located between the primers is amplified exponentially. The rest of the DNA in the genome is not amplified and remains invisible in the gel.

Primer design

Some simple rules aid in the design of efficient primers:

1. Typical primers are 18 to 28 nucleotides in length having 50 to 60% G + C composition. 2. The calculated melting temperatures for a given primer pair should be balanced. One can use the rule-of-thumb calculation of 2°C for A or T and 4°C for G or C. Depending on the application, melting temperatures between 55°C and 80°C are desired.

3. To prevent self-annealing, primers should not be complementary. This pre-caution is critical at the extreme 3' ends where any complementarity may lead to considerable primer-dimer formation and reduces the yield of the desired product.

4. Runs (three or more) of C's or G's at the 3' ends of primers may promote mispriming at G + C-rich sequences and should be avoided when possible.

If all else fail, it usually helps to try a different primer pair. A less obvious reason for some primers failing to work is the presence of secondary structure in the template DNA. Software is also available from many commercial and academic sources to assist in the process. Most software packages for DNA sequence analysis now include menus for PCR primer design.

Nested PCR

Nested PCR primers are ones that are internal to the first primer pair. The larger fragment produced by the first round of PCR is used as the template for the second PCR. Nested PCR can also be performed with one of the first primer pair and a single nested primer. The sensitivity and specificity of both DNA and RNA amplification can be dramatically increased by using this method. The specificity is particularly enhanced because this technique almost always **eliminates any spurious nonspecific amplification products**. This is because after the first round of PCR, any nonspecific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as a template for further amplification, thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme sensitivity.

RNA (RT) PCR

PCR amplifies DNA sequences. In order to perform PCR on RNA sequences using *Taq* DNA polymerase, the RNA must first be transcribed into a cDNA (complementary DNA) copy of the RNA sequence because *Taq* has limited reverse transcriptase activity. This is called reverse transcription (RT). Thus, RNA amplification is achieved by the reverse transcription-polymerase chain reaction (RT-PCR). There are several different kinds of primers that can be used to make cDNAs, like oligo-dT will prime cDNA synthesis on all polyadenylated RNAs, random-primed cDNA synthesis gives a broad range of cDNAs and is not limited to polyadenylated RNAs and lastly, oligo-nucleotide primers complementary to the RNA(s) of interest may be used to synthesize highly specific cDNAs.

One-tube RT-PCR incorporates both the reverse transcriptase enzyme and a thermostable DNA polymerase in a single tube for synthesis and amplification of the target RNA sequence. This is the preferred procedure for routine analy-sis. Commercial RT-PCR kits are available and alternatively, reagent mixes can be prepared also from separate component parts.

Problems with PCR

PCR is an extremely powerful technique, but its very power can also lead to considerable problems, particularly when detecting virus or bacterial genes for diagnostic purposes. It is important to remember that nucleic acid from dead as well as viable microorganisms will give a positive reaction. Since even a single molecule of DNA can be amplified by PCR, it is also vital to prevent cross-contamination of DNA samples with amplified or foreign DNA. The slightest contamination of glassware, pipettes or reagents can result in the production of false-positive reactions. Such contamination problems impose a need for extreme cleanliness and rigorous controls. Amplification reactions should be performed in physical isolation (i.e. in a different room) from the parts of the laboratory where specimens are received and target nucleic acid is prepared. Various techniques for reducing extraneous DNA contamination of PCR products have been described, but it is vital that each set of PCR amplifications should include control reactions to verify the purity of reagents and the cleanliness of equipment.

PCR is vulnerable to contamination that will cause erroneous results. False positives will result from contamination of the reaction with target RNA or RT-PCR products. False negatives can be caused by the presence of inhibitors in the test samples or badly degraded target materials. For competent PCR, the sample must be either fresh and in good condition prior to nucleic acid extrac-tion or preserved to maintain nucleic acid suitable for extraction. Proper con-sideration to the extraction procedure is also important. Maintenance of rigor-ously clean experimental techniques, use of standard reaction conditions and inclusion of internal standards as positive and negative controls are essential to gain accurate interpretation of the results.

Other problems may arise from the relatively high error rate of *Taq* polymerase. Base substitutions occur at about one in every 9,000 bp, and frameshifts at about one in every 40,000 bp. Although such error rates may seem to be insignificant, they may have profound effects on the homogeneity of the amplified products.

Although PCR can now be semi-automated because of the availability of the thermocycler, the technique still requires a certain amount of technical skill and some specialized equipments to prepare samples and perform amplification reactions successfully.

SUMMARY

The improvement of existing immunoassay techniques, development of monoclonal antibody technology and the development of new immunoassay approaches are all working together to provide new tools for the detection of disease-causing organisms in fish and crustaceans. Following the introduction of nucleic acid hybridization technique and PCR, it was recognized that the methods offered a sensitive approach to the detection and identification of specific microorganisms as in the case of a bacterial or viral infection in a variety of sample types. Potentially, a characteristic DNA sequence from a single virus particle or cell of a particular organism can be amplified to detectable levels within a short period of time. Conventional diagnostic methods that involve the culture of microorganisms can take days or weeks to complete or very tedious to perform. PCR offers a rapid, very sensitive, very specific and simple alternative. Further developments in immunodiagnostics and emerging technologies such as DNA-based tests will revolutionize the detection and identification of infectious disease agents.

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