Winter School on

'RECENT ADVANCES IN DIAGNOSIS AND MANAGEMENT OF DISEASES IN MARICULTURE'

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Course Manual

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PCR BASED DIAGNOSTIC TECHNIQUES

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Introduction

Use of PCR assisted diagnosis has many advantages over the conventional methods. Since PCR can amplify even a single strand of DNA into millions of copies within hours. even a pathogen collected from the infected fish could be used directly for the identification. Thus. considerable time can be saved.

Two of the PCR based techniques merits special mention for their use in rapid detection of microbial pathogens.. They are the DNA fingerprinting based on arbitrarily primed PCR (AP PCR) and amplification of species specific genes of the pathogens using specific primers flanking these genes. Before going into the details of these techniques let us examine how the PCR works.

A. The Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a technique for *in vitro* chemical synthesis of multiple copies of a given DNA by performing successive rounds of *in vitro* nucleic acid replication. This is achieved by using two oligo-nucleotide primers that hybridize (anneal) to the opposite strands of the target DNA at positions that flank the region to be amplified and synthesizing of the intervening portions of both the strands through simultaneous extension of both the primers. A repetitive series of cycles involving template de-naturation, primer annealing and extension of the annealed primers by DNA polymerase results in the exponential accumulation of the DNA whose termini are defined by the 5' ends of the primers. Since the primer extension products synthesized in one cycle can serve as a template for the next, the number of target DNA copies approximately doubles at every cycle. Thus, 20 cycles of PCR, yields about a million fold amplification.

The tremendous applications of PCR were possible because of the availability of nucleic acid sequence information. The components of PCR reaction viz: template (the DNA to be amplified), primers.Taq DNA polymerase, the de-oxyribonucleoside tri-phosphate and buffer containing magnesium are assembled in a tube and the amplification reaction is carried out by cycling the temperature within the reaction tube. For any given pair of oligonucleotide primers, the optimal conditions of all the above ingredients and parameters have to be standardized. Eventhough, there is no single set of conditions that will be optimal for the reaction, the conditions outlined below defines a common starting point from where modifications can be attempted.

Materials and Reagents for a standard PCR

The Primers

Oligonucleotide primers in the range of 18 to 30 bases are generally used for the conventional PCR. The sequence of the primers should be complimentary to the 3' ends of the target (template) DNA strands to be amplified. Primers are the most important components that determine the success of an amplification reaction. Though there is no

set rules that will ensure the synthesis of an effective primer pair, the following guidelines are useful.

(a) Wherever possible, select primers with a random base distribution and with a GC content similar to that of the fragment being amplified. Avoid primers with stretches of polypurines, polypyrimidines or other unusual sequences.

(b) Check the primers against each other for complementarity. Use primers with low complementarity to each other. Avoid primers with 3' end overlaps in particular. This will reduce incidence of "primer dimers".

Most primers are generally 18 to 30 bases in length and the optimal length to be used in amplification will vary. Longer primer may be used but are seldom necessary.

If shorter primers or degenerate primers are used, the thermal profile should be modified considering the lower stability of the primed target. However, the 3' end of the primer should match the template exactly. Generally, concentrations ranging from 0.05 to 0.5 μM of each oligonucleotide should be used.

The Reaction Buffer

The components of PCR buffer, particularly the concentration of $MgCl_2$ have a profound effect on the specificity and yield of an amplification product. Concentration of about 1.5 mM is usually optimal. Excess Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield. Several successful buffer formulations have been published.

The recommended PCR buffer should contain 10-30mM Tris-HCl (pH 8.4) also. KCl up to 50mM can be included in the reaction mixture to facilitate primer annealing. Excess KCl inhibits Taq polymerase activity.

Gelatin or bovine serum albumin (100 μg/ml) and a non-ionic detergents such as Tween 20 and NP40 (0.05 - 0.1%) are included to help stabilize the enzyme. The nonionic detergents can be replaced by 0.1% Triton X-100, but some detergent is essential.

Deoxynucleoside triphosphate

The deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP) are generally used at concentrations of 100-200 μM each. Higher concentrations may lead to misincorporations. Low dNTP concentration reduces mispriming at non target sites. The lowest dNTP concentration appropriate for the length and composition of the target must be standardized.

Taq polymerase

The required concentration of Taq DNA polymerase concentration is between 1 and 2.5 units per 100 μl reaction when other parameter are optimum. When optimizing a PCR, enzyme concentration ranging from 0.5 to 5 units/ 100 μl are tested and results assayed by agarose gel electrophoresis. If the enzyme concentration is too high, non-specific background products may accumulate and if too low, an insufficient amount of desired product is made.

Thermal Cycle for PCR

Various ingredients for the PCR are mixed in the required concentrations and the different events in the PCR are carried out by manipulation of the temperature of the PCR mix in a cyclic manner. A thermocycler can give the required temperatures in the required order for the specified duration.

Primer annealing

The temperature and length of time required for primer annealing depends upon the base composition as well as the length and concentration of the primers. As a rule of thumb. annealing temperature of $5^{O}C$ below the true Tm of the primers can be attempted. Annealing temperature in the range of 5o to 60 ^{O}C generally yield good results. At the optimal primer concentration, annealing will require only a few seconds.

Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces misextension of incorrect nucleotides at the 3' end of the primers. Therefore, stringent annealing temperature, especially during initial few cycles will help to increase specificity.

Primer Extension

Extension time depends on the length and concentration of the target sequence and the temperature. Primer extensions are usually performed at $72^{O}C$. The rate of nucleotide incorporation at $72^{O}C$ vary from 35 to 100 nucleotides per second depending upon the buffer, pH, salt concentration and nature of the DNA template. An extension time of one minute at $72^{O}C$ is considered sufficient for products up to 2 Kb in length.

Denaturation

Typical denaturation temperature is $94^{O}C$ for 30 sec. Higher temperatures eg. $97^{0}C$ may be necessary for G+C rich targets. Denaturation steps that are too long or too high lead to unnecessary loss of enzyme activity.

Cycle number

The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimal. Too many cycles may increase the amount and complexity of non specific background products. Too few cycles give a low product yield.

Detection and analysis of PCR product

The PCR product will be a DNA molecule of defined length. The simplest way to check this is to load a portion of the PCR product and molecular weight markers into an agarose get containing ethidium bromide and carry out an electrophoresis. The DNA fragments generated by the PCR should be readily visible over an ultraviolet transilluminator.

B. Detection of pathogens through amplification of species specific genes

All the pathogens possess certain genes mainly related to its virulance factors or toxins, which are specific to that pathogen. The core sequence of these genes is usually highly conserved. Therefore, an ideal approach for the identification of the suspected pathogen is to amplify such genes through PCR, using primers synthesized to have complementarity to the conserved regions of these genes. This implies that previous knowledge of the sequence of the species-specific gene should be available. With the

increasing availability of information on the sequences of such genes of the common pathogens, application of this method shall become more common.

The amplification of the conserved regions of the DNA/RNA of the viral pathogens using appropriate primers is generally used for their detection. Published sequence information of the cloned DNA of various viral pathogens are available for the synthesis of primers for their detection.

Given below is the list of specific genes of certain bacterial species that could be utilized for their identification using PCR.

Bacterial Species	**Gene**
Aeromonas salmonicida	vapA (Virulance array protein A) gcat (Glycerophospholipid : cholesterol acetyltransferase gene)
Aeromonas hydrophila	ahcytogen (Cytolytic enterotoxin gene)
Vibrio vulnificus	cth (Cytotoxin haemolysin gene)
Vibrio cholera	ctxA (Cholera toxin gene) ctxB
V. parahaemolyticus	gyrB (Gyrase B gene) tdh (Thermostable direct haemoysin gene)
Listeria monocytogene	dth (Delayed hypersensitivity factor) iap (Invasion associated protein gene) in1 A (Internalin protein gene)
Yersina enterocolitica	**YadA (Virulence associated outer membrane protein)**
Luminous bacteria	LuxA (Luciferase gene) Lux B

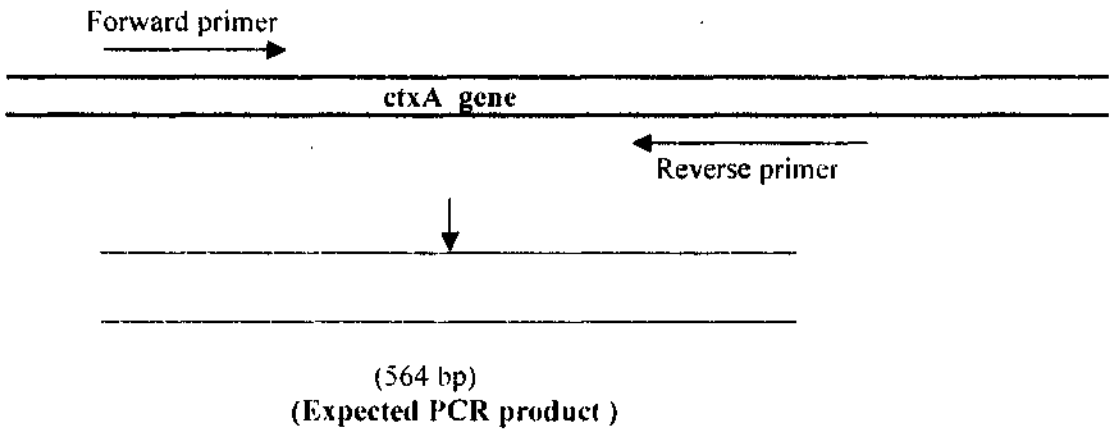
The potential application of these genes for the identification of the bacterial species using PCR have been demonstrated by different workers.

For example a 300 bp fragment of dth-18 gene of *Listeria monocytogenes* amplified by Fluit *et al.* (1993) using two 20 bp primers was found to be useful for its identification. These primers were

Forward : 5' - CTA ATC AAG ACA ATA AAA TC - 3' and
Reverse : 5' - GTT AGT TCT ACA TCA CCT GA - 3'

Similarly, a 564 bp fragment of the ctxA gene of *Vibrio cholerae* amplified by Fields *et al.*, (1992) using two primers of 21 bp each was claimed to be useful in identifying *V. cholerae* bacteria.

PCR amplification of ctxA gene segment of V. Cholerae



(564 bp)
(Expected PCR product)

The sensitivity and specificity of the PCR can be further enhanced manifold by carryin0g out nested PCR. The process utilizes two consecutive PCRs, each usually involving 25 cycles of amplification. The first PCR utilizes a pair of primes flanking the gene in question while the second PCR uses another pair of primers having complementarity to an internal segment of the gene which was amplified in the first PCR. The larger fragment produced by the first reaction is used as the template for the second PCR. The nested PCR will be more sensitive than standard PCR. Therefore, when information on the sequence of species specific genes are available, amplification and visualization of that gene using a nested PCR is the method of choice when viewed from the point of sensitivity and reliability.

C. Application of DNA fingerprints of pathogens for their detection

DNA fingerprinting based on randomly primed PCR/arbitrarily primed PCR is a rapid and sensitive technique that can be employed for distinguishing species and strains of bacteria. This rapid method for fingerprinting of genomic DNA described in 1990 simultaneously by Welsh & Mc Clelland as well as by Williams and coworkers involves the use of random primers for DNA amplification. It is modified form of PCR. It varies from standard PCR in the primers being used. While in the standard PCR the two primers being used at a time which are known to flank the desired portion of the target DNA, in the arbitrarily primed PCR , only one primer is used at a time with out any prior knowledge as to whether it is flanking any particular portion of the template DNA. It therefore , tires to utilize the chance occurrence of sequences on the target DNA which are complimentary to the randomly selected primers. If the primer locate complementary sequences on both the strands of the target DNA , then they anneal at the sites and under appropriate PCR conditions amplify the portion lying between two annealed sequences . The number of amplified segments depends on the number of complementary sites on the target DNA for the primer. The arbitrarily primed PCR products display differences in band patterns , if the two genomic DNAs used as templates are different. This is the underlying principle in the use of arbitrarily primed PCR for fingerprinting to distinguish strains and species of pathogens. The band patterns resulting from AP PCR against an array of different primers are prepared for each species and strain of a known pathogen to be later used as a frame of reference for comparing the patterns obtained with PCR of suspected pathogens.

One major advantage of this type of fingerprinting is that the previous knowledge of the sequence of the template DNA is not at all required . Other advantages are, the

rapidity, high sensitivity and cheapness compared to other techniques like southern blotting probes. Use of this method for identification of species and strains of bacteria *viz. Staphylococcus* and *Streptococcus* (Welsh & Mc Clelland, 1990) *Listeria* (Mazurier & Wernars, 1992) *Borrelia* (Wesh *et al.*, 1992) and *Vibrio (* Martinez *et al.*, 1994) has been demonstrated.

Duplex PCR Screening of White spot Baculovirus (WSBV)

White spot disease caused by White spot Baculovirus (WSBV) is on of the major threat faced by the shrimp farming industry. As there is no cure for this disease, stocking disease free larvae is one of the management measures recommended to prevent critical transmission of the virus. This calls for a cheap, simple, sensitive and rapid detection methods.

Various kinds of molecular diagnostic kits are being marketed for this purpose, among which PCR based detection is the most sensitive and very useful for the pro-active disease management. The nested PCR kits are mainly being used currently.

We have at CMFRI has designed a duplex PCR for detection of WSBV which is cost effective, faster and reliable compared to the nested PCR. This involves the simultaneous PCR screening of two different regions of the viral genome with appropriate primers .

The duplex PCR has the following advantages :

Rapidity : While nested PCR is carried out in two stages, duplex PCR is conducted in a single run, thus, reducing time required for the screening.

Cost effectiveness : Since the assay volume and constituents used in the duplex PCR is equivalent to the first run of the nested PCR , it is less expensive.

Reliability : Since two different regions of the viral genome are amplified and checked simultaneously , it has got high reliability also.

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DISEASE DETECTION USING PCR
(White Spot Syndrome Virus Detection Kit of CMFRI- Duplex PCR assay)

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I. Template DNA preparation

1. Thoroughly wash the shrimp with distilled water

2. Wear gloves and carefully remove about 30 mg gill tissue using a sterile forceps into a sterile microfuge tube supplied.

3. In case of larvae and post larvae, aseptically transfer about 25 numbers of larvae (30 mg) / 10 to 15 numbers of post larvae (30 mg), into the microfuge tube.

4. Homogenize the sample using 1 ml of homogenizing solution and disposable pestle supplied as described below. Initially the sample may be crushed in 200 µl (0.2 ml) of the homogenizing solution and make up the volume to 1 ml and thoroughly homogenize. Discard the used homogenizer after autoclaving.

5. Tightly close the tube containing the homogenate, cover the lid with parafilm and insert pin prick hole on the lid.

6. Arrange the tubes on a hard thermocol float and boil for 10 minutes.
7. Allow the sample to cool to the room temperature.

8. Centrifuge the tube at 10000 rpm for 15 minutes in a centrifuge.

9. Carefully recover the supernatant containing the DNA template using a micropipette, transfer into another sterile microfuge tube and store at –20° C till used for PCR.

II. Polymerase Chain Reaction (PCR)

1. Arrange the sterile 0.2 ml PCR tubes supplied in an aseptic environment and label properly.

2. Take out the PCR pre-mix and Taq DNA polymerase supplied as well as the sample supernatant containing the DNA template and keep on ice.

3. Carefully pipette out **23.6 µl** of PCR mix into a PCR tube using micropipette (aerosol tips are ideal)

4. Add **0.4 µl** of Taq polymerase and **1 µl** of the sample supernatant into the PCR tube.

5. Tightly close the PCR tube and ensure that no air bubbles are trapped inside the mix. Mix the contents of the tubes by gently tapping with fingers or by spinning in a mini microfuge tube at 6000rpm for 15-20 seconds. (Similarly arrange for the PCR with the positive and negative DNA samples also)

Note: If more samples are to be screened, then prepare a master mix as follows.

* Determine the total number of PCRs to be performed by adding **one** positive control and **one** negative control **plus one** to the **number of samples to be tested**.(ie, Number of sample to be screened + three)

* Calculate the volume of ingredients to be added for the master mix as follows.

  Volume (µl) of pre mix = 23.6 x total number of PCRs to be performed.

  Vol. (µl) of Taq Polymerase = 0.4 x total number of PCRs to be performed.

* Prepare the Master mix by carefully pipetting out the above volumes into a 2 ml sterile microfuge tube.

* Aliqout 24 µl from the above master mix into each of the PCR tubes . Add 1µl template DNA into respective PCR tube.

* Add 1µl of the Positive control DNA into the Positive control PCR tube.

* Add 1µl of the negative control DNA into the negative control PCR tube .

6. Load the PCR tube into the thermocyler and set the PCR as follows.
   - Initial denaturation: 95°C for 3 miutes
   - Followed by 35 cycles of:
     95°C for30 seconds
     58°C for 60 seconds
     72°C for 60 seconds
   - Followed by final extension at 72°C for 5 minutes

   (To select virus free larvae / post larvae etc. for stocking, 40 cycles are advisable)

III. Detection of the PCR product

Detection of PCR product is carried out by 1.5% agarose gel electrophoresis.

- Sample preparations for electrophoresis:

a) Mix 5µl of the PCR product and 2µl of the loading dye supplied. (Prepare for each of the PCR sample, positive control and negative control)
b) Mix 5µl of the DNA marker supplied and 2µl of the loading dye

- Cast a 1.5-% agarose gel and load 7µl of the above sample + dye mixture into the well of the gel. Load each sample into a separate well. Load the marker, positive control and negative control in separate wells and carry out electrophoresis at a constant voltage of 80.After the run is over, stain the gel with ethidium bromide(1µg /ml) and visualize the bands on UV transilluminator.

III. **Interpretation of result**

Presence of virus in the sample is indicated by two bands of 0.95Kb and 0.64Kb resolved in the gel.