Polymerase chain reaction (PCR) in disease diagnosis

By

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Various molecular biology techniques continue in becoming more important in fish and shrimp farming, particularly in detection and prevention of various diseases. One of the most prominent techniques is the Polymerase Chain Reaction (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 (messenger ribonucleic acid), rapidly synthesize, clone and sequence virtually any segment of DNA (deoxyribonucleic acid). Despite the incredible power of the technique there has been one major limitation, that is, the DNA must be extracted from the sample. Thus one can not correlate PCR results with the pathological features of the material being tested.

The development of PCR means that small amount 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 prevention, 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 thermo-stable 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:

(1) **melting or denaturation** (strand separation) of the double strand DNA (one to several minutes at 94-96ºC). The PCR reaction requires a single-strand template. The first step denatures or melts the double-strand template DNA so that all the DNA is single-strand. This allows the oligonucleotide primers to anneal to the single-strand template DNA at specific locations. Temperature of 94ºC for 30 seconds seems to work well, but shorter times have also been recommended. Remember, if the melting temperature is too low or time is too short the double-strand DNA may not denature thereby reducing the efficiency of the reaction. This is especially true for the first cycle in which the goal is to denature high molecular weight DNA. Some protocols suggest a long initial denaturation. On the other hand, the enzyme Taq polymerase will become less active after repeated denaturation cycle so, there is a need to balance between denaturation of the DNA and of the enzyme;

(2) **annealing** of the two primers to opposite DNA strands (one to several minutes at 50-65ºC). Once the template DNA has been denatured, the temperature must be lowered to a level that allows the primer to anneal. The trick is to lower the temperature to a level that allows the primer to anneal to the complementary sequence, if the temperature is too low the primer will sit down randomly (non-specifically) and if too high the primer will not sit down at all. Standard temperature seems to be about 55ºC for 30-60 sec; and

(3) **extension** of the primers by polymerase-mediated nucleotide additions to produce two copies of the original sequence (one to several minutes at 72ºC). The Taq polymerase works best at temperatures between 72-75ºC and so we raise the temperature from the relatively low annealing temperature to a temperature at which the Taq polymerase can function efficiently. The polymerase has to add nucleotides to the 3' end of the primer sequence annealed to the template DNA (please see figure below). The primers are necessary for the initiation of the reaction. The template DNA acts as a reference strand for the polymerase which adds the complementary nucleotide bases starting at the position just after the 3' end of the primer sequence (ADENOSINE pairs with THYMINE and GUANINE pairs with CYTOSINE). The primers are incorporated into all subsequently amplified DNA templates insuring perfect priming sites in subsequent PCR cycles.

PRIMER	5' TACAGCGCCACGTTA 3'
TEMPLATE	3' ATGTCGCGGTGCAATGATGCGTACGTAATGA 5'

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 _M each
Primer 1	20 pmol	
Primer 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 theoretical 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:

Typical primers are 18 to 28 nucleotides in length having 50 to 60% G+C composition.

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.

To prevent self-annealing, primers should not be complementary. This precaution 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.

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 analysis. 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 extraction or preserved to maintain nucleic acid suitable for extraction. Proper consideration to the extraction procedure is also important. Maintenance of rigorously 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.

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the aquasilviculture trials in collaboration with local government units, other activities in the Aklan mangrove sites are the survey and mapping of the 75-ha area in Ibajay, construction of a treehouse, and the educational use as field site by Coastal Resource Management trainees (of SEAFDEC-AQD) and field biology students (of the University of the Philippines in the Visayas).

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Abstract. The effect of scheduled use of high-protein and low-protein diets on body weight and fry production of Nile tilapia, *Oreochromis niloticus* (L.), was determined. A preliminary feeding trial was first conducted on fingerlings. These were fed a high-protein diet (H, 25% protein) or a low-protein diet (L, 18% protein) daily, or diet H for 1-3 days followed by diet L for 1-4 days. Final body weight was significantly higher (P <0.05) in fish fed diet H daily and in fish fed diet H for 2-3 days followed by diet L for 1 day (2H-1L and 3H-1L). Fingerlings on 1H-1L and 3H-2L had slightly lower growth. Based on the response of the fingerlings, five feeding schedules were tested with the broodstock. A high-protein diet (HP, 40%) and a low-protein diet (LP, 25%; same as H for fingerlings) were used. Feeding schedules significantly influenced body weight of female but not the male fish. Fry production was not significantly affected by the feeding schedule for broodstock. When growth, fry production and saving in feed cost were all considered, the broodstock on 1HP-1LP and 3HP-2LP feeding schedules both gave the highest overall performance. These findings give fish farmers an option in the management of feeding of tilapia broodstock.

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Abstract. In order to investigate the effect of different protein/energy levels of diets (two commercial and one laboratory) on voluntary feed intake and energy partitioning in Tilapia (*Oreochromis niloticus*), 15 fish with an initial body mass of 33 g were reared individually in respirometric chambers for 42 days and offered 3 diets *ad libitum*. The protein contents of the diets were 36.1, 33.8 and 36.8% (dry matter base); the energy content 18.9, 18.4 and 19.2 kJ GE/g and 11.7, 10.5 and 15.4 kJ ME/g. The initial body composition and energy content was estimated from a control group. Feed consumption was recorded for each individual fish. Body mass development was monitored weekly. At the end of the experiment, the fish were sacrificed and their chemical composition (protein as N.6.25, lipid, ash) and gross energy content determined. To establish energy budgets, ingestion (I) was calculated from feed intake, retention (P) from accretion in the carcass, heat production (R) from oxygen consumption (indirect calorimetry) and apparently non-utilised energy (faecal and non-faecal losses, U) by difference from energy ingestion. In the beginning, food consumption amounted to approximately 5% body mass equivalent (BME) per day for all groups and gradually decreased to 2.5, 2.8 and 1.6% BME by the end of the experiment. While the food consumption was significantly different between the treatments, there were no significant differences in the body mass development. Average final body mass was 98.6, 93.8 and 103.7 g. Energy retention was 29.7, 29.2 and 44% of GE ingested; heat dissipation 32.1, 27.9 and 36.0%; faecal and non-faecal losses 38.2, 43.2 and 19.6%. For all energy budget parameters, values for the laboratory diet were significantly different from those of commercial feeds 1 and 2. Calculation of metabolisable energy from ingested feed revealed no significant differences in the energy uptake, suggesting that the voluntary feed uptake was controlled by the metabolisable energy. The fish were able to completely compensate for the lower ME content of the commercial feeds by increasing voluntary feed intake.

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