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Winter School on

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

7th to 27th November, 2002

Course Manual

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MOLECULAR BIOLOGICAL TECHNIQUES IN DISEASE DIAGNOSIS

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Rapid diagnosis of diseases is indispensable in aquaculture management for control of diseases and for developing appropriate strategies in health management of cultured stock. Lack of knowledge about diseases and their reasons as well as absence of tools to accurately diagnose diseases often lead to indiscriminate use of chemicals and antibiotics in culture systems. This results not only in environmental pollution but also pave the way for the development of disease resistant pathogenic organisms (Karunasagar and Karunasagar, 1998). Rapid diagnostic methods of pathogens would facilitate in

- Surveillance and monitoring programs
- Assessment of efficacy of treatment regimes
- Identification of sources of contamination
- Screening broodstock and larvae in hatcheries

Conventional methods of disease detection are time consuming and lack sensitivity. These two weaknesses are overcome with molecular diagnostics. Since early 1980s, there has been steady increase in application of DNA-related techniques in the diagnosis of infectious diseases caused by bacteria, virus, fungus and parasites. Rapid, pathogen-specific diagnostics would be particularly appropriate for disease management and control when diseases emerge in new geographic locations or host species. An additional application for molecular techniques is for research into pathogenesis of a disease via non-lethal sampling, e.g. of haemolymph, fin- or gill-clips (McGladdery, 2000).

The present paper is an attempt to briefly outline DNA-related techniques used in diagnosis of aquaculture stocks. Discussion on the specific cases molecular diagnostics in shrimps and other cultured organisms is beyond the scope of this paper. Useful reviews are available elsewhere (Karunasagar and Karunasagar, 1998: Lightner and Redman, 1998; Walker and Subasinghe, 2000).

DNA diagnostics

DNA diagnostics of diseases is broadly classified into DNA probe hybridization and Polymerase Chain Reaction (PCR). Most important basic requirement for both approaches is DNA extracted from any convenient tissue of the organism to be screened for disease. A variety of techniques, such as alkaline lysis, proteinase K digestion with phenol:chloroform extraction, treatment with guanidinium salts (passive) and boiling are available. It is important that a good DNA extraction protocol should be ideally rapid, non-toxic, economical, and have low risk of contamination.

Nucleic acid probes and hybridization technique

Nucleic acid probes are segments of DNA or RNA that have been labeled with enzymes, antigenic substances and chemiluminescent moieties or radioisotopes. They would bind with high specificity to complementary sequences of nucleic acid to form double stranded molecules and this process is known as *hybridization*. Probes can be directed to either DNA or RNA targets and can be 20 to 20,000 bases long.

Oligonucleotide probes (generally less than 50 base pairs in length) have the advantage of hybridizing more rapidly to target molecules and can be chemically synthesized and purified by instruments available commercially.

During the early days of DNA probes, ^{32}P label was most commonly used. In view of health hazards paused by radioactive substances, non-radioactive probes have become more popular in recent times. Chemiluminescent labels, such as biotin and digoxigenin have sensitivity ranges equal to or more than ^{32}P labeled probes. Further, longer shelf lives and non requirement of additional safety and control measures make them more acceptable. DNA probes are presently available to detect several penaeid shrimp viruses including infectious hypodermal and hemopoietic necrosis virus (IHHNV), hepatopancreatic parvo-like virus (HPV), monodon baculovirus (MBV), baculovirus penaei (BP), white spot baculovirus and taura syndrome virus (TSV) (Lightner and Redman, 1998).

Essentially, nucleic acid hybridization involves four steps:

- ❖ Transfer and immobilization of DNA on a membrane, such as nylon/nitrocellulose (blotting)
- ❖ Prehybridization incubation of the blot
- ❖ Hybridization – incubation of the probe with target DNA on the blot
- ❖ Post hybridization washing

Factors influencing hybridization

A number of factors influence rate of hybridization as briefly mentioned below,

i. Probe concentration – Generally, more the probe concentration more the rate of hybridization.

ii. Complexity of probe sequence – Complexity is judged by the number of unique nucleotide sequences, e.g. if the probe has more number of tandem repeats of specific sequences, rate of hybridization would be lower.

iii. Probe size – Rate of hybridization is significantly reduced with an increase in probe size and this effect is even more pronounced if the probe used in double-stranded compared to a single stranded one.

iv. Ionic strength/salt concentration – The rate of hybridization is directly proportional to the ionic strength of hybridization medium.

v. Temperature and pH – For well matched hybrids, the ideal range of temperature is 65-68^{0}C (in aqueous solution) and for poorly matched hybrids, the range is 35-42^{0}C. pH do not have any effect on the rate of hybridization between the range of 5 to 9.

vi. Presence of inert polymer – Inert polymers, such as Dextran Sulfate can increase the hybridization signal, though it can result in high non-specific background.

***In situ* hybridization**

In situ hybridization can be carried out on chromosomal preparation, frozen sections, cytological specimens, touch preparations or paraffin sections. DNA or mRNA sequences can be detected.

The sections are treated with a protease to allow penetration of the probe. Before hybridization, the cellular DNA in denatured by heating. In order to minimize non-specific staining, the reaction mixture containing the denatured probe should have an excess of unlabelled "carrier" DNA. This will block the non- specific binding sites in the section. Radio labeled, digoxigenin labeled, or biotinylated probes may be used. Methods

The chief advantages of this technique over southern hybridization are that no steps involving with restriction endonuclease enzymes, gel electrophoresis or transfer from gel to membrane are needed and may be used to screen multiple samples. Further, the technique is more sensitive.

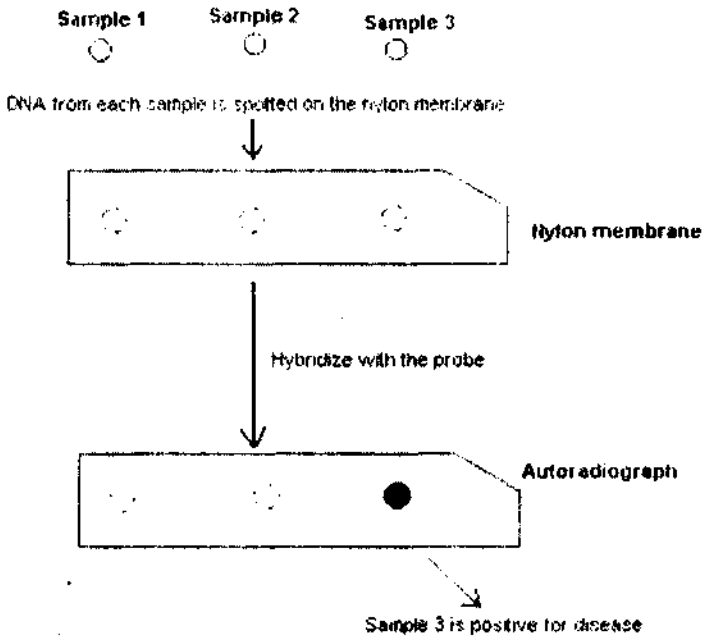

Fig.1 Dot-blot hybridization to detect disease

The four steps involved in dot blot technique are,

a. Spotting of membranes – Denatured DNA is spotted on nylon membrane, irradiated on a UV transilluminator and can be stored at 4^0C till next step.

b. Labeling of probe – Either radioactive method or non-radioactive method.

c. Hybridizing labeled DNA to immobilized target DNA.

d. Detecting probe DNA-target DNA hybrids – Autoradiography or chemiluminiscent method.

Sometimes, an apparatus is used for placing spots on the membrane, through slots made in this equipment. The spots made thus are in the form of oblong slots rather than round blots. These slots are used just like dot blots and are described as slot blots (Fig.2).

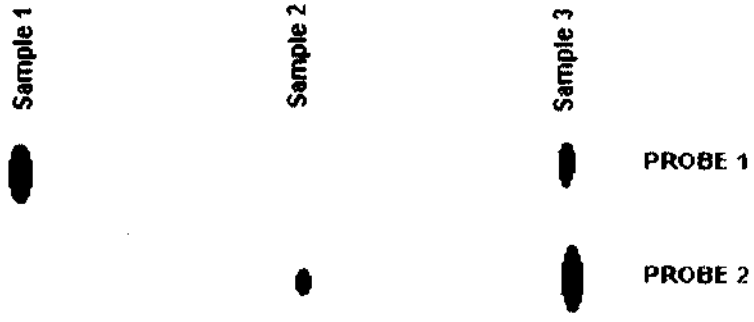

Fig.2 Schematic representation of Slot blots from genomic DNA of 3 samples showing difference in abundance of sequences related to two different probes

for visualizing non-radioactive probes takes only a few hours while procedures incorporating radio labeled probes take much longer time.

This technique is particularly useful for demonstration of viral DNA and RNA *in situ*. It is also applied in other situations, such as the demonstration of mRNA transcripts of various genes (e.g. light chain genes, tumour marker genes, drug resistance genes, etc.) within the cells.

Blotting

Due to malleable nature of gel it is difficult to probe the electrophoresed DNA fragments on the gel surface. Hence it is imperative to transfer DNA before probing to a solid surface and immobilize. This transfer process is also known as *blotting*. Transfer technique should ensure the true replica of resolved fragments in gel should be obtained on membrane. All transfer techniques consists of five basic steps, as briefly stated below,

- Fragmentation – Done to ensure maximum quantitative transfer of especially large DNA molecules to the membrane.
- Denaturation - Essential to denature the DNA (before fixation on the blot) so that it can hybridize subsequently, with the probe sequences.
- Neutralization - DNA should be in neutral solution so that it can efficiently bind to nylon or nitrocellulose filters and hybridize with the probe.
- Transfer - There are three methods, (a) capillary transfer or passive diffusion as is done in Southern blotting, (b) electroblotting, in which the driving force for the fractionated nucleic acid molecules is the electric field and (c) vacuum blotting, in which the nucleic acid molecules are drawn from the gel by applying vacuum from below.
- Immobilization – Immobilization of blotted DNA is achieved by vacuum baking at 70-80^{0}C or UV-cross linking depending on the type of filter used.

Southern blot, named after its inventor (Southern, 1975) and dot blot hybridization techniques are two of the most important blotting techniques. The latter is more rapid and thus more applicable for quick diagnosis of diseases in cultured stocks. Details of southern blotting are given elsewhere (Brown, 1990; Old and Primrose, 1994).

Dot blot/slot blot hybridization technique

In this technique, isolated unrestricted and denatured DNA is spotted on to the membrane (nylon/nitrocellulose), subsequently fixed by baking/UV cross linking and hybridized. Radiolabelled, Biotin labeled or Digoxigenin labeled probes may be used in conjunction with the appropriate detection system (Fig. 1).

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Polymerase Chain Reaction (PCR)

PCR is an in vitro method of enzymatic selective amplification of a chosen region of a DNA molecule (Fig. 3).

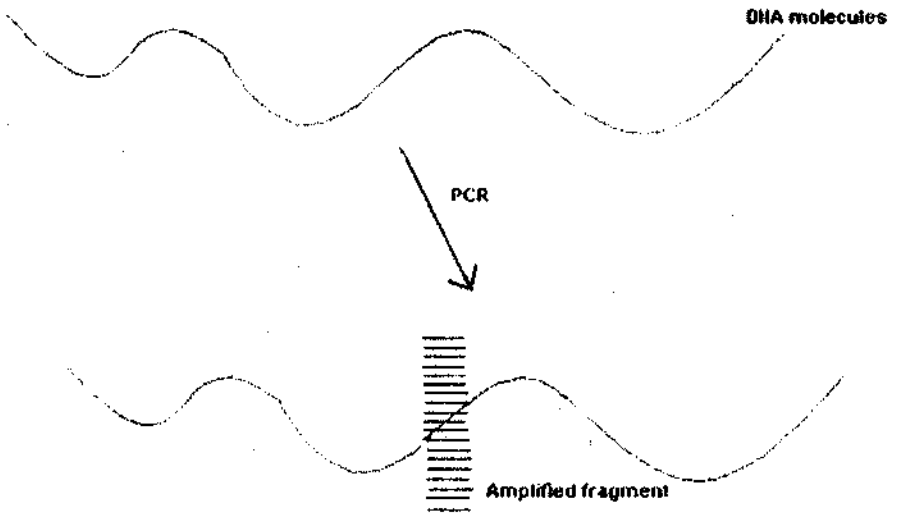

Fig.3 Amplification of a specific region of a DNA molecule by PCR

The concept of PCR was developed by Karry Mullis, R.K. Saiki and others during 1980s (Saiki et al., 1988). The method consists of repetitive cycles of DNA denaturation, primer annealing and target DNA extension with the help of a thermostable DNA polymerase

.Two oligonucleotide primers are allowed to anneal with heat denatured target DNA. The target DNA sequence is then allowed to polymerize with *Taq* DNA polymerase (obtained from *Thermus aquaticus*, a bacterium naturally occurring in hot springs). The two primers hybridize to opposite strands of the target sequence such that synthesis proceeds across the region between the primers, replicating that DNA segment. These steps of denaturation, annealing and extension are repeated many times. The product of each PCR cycle is complementay to and capable of binding primers, and so the amount of DNA synthesized is doubled in each successive cycle.

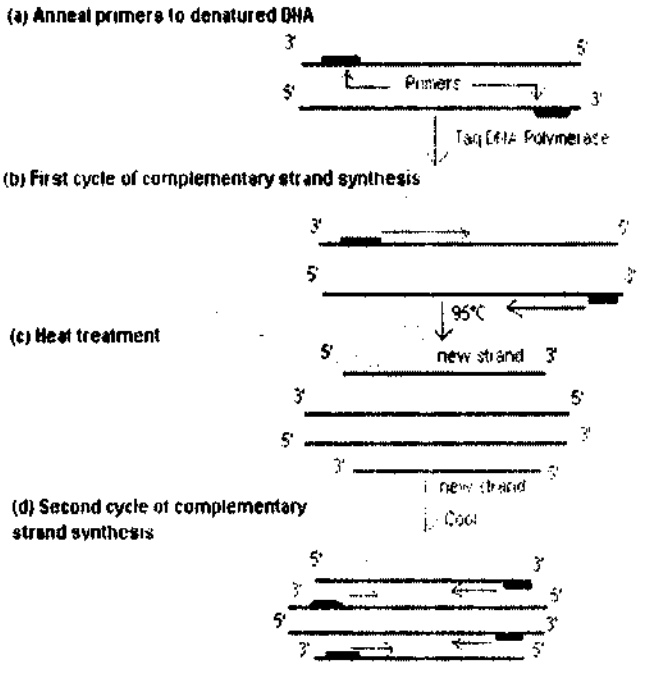

Fig.4 Schematic diagram of polymerase chain reaction (PCR)

The amplification takes place at the rate of 2^n, where 'n' is the number of cycles (Fig. 4). The original template DNA can be in a pure form and as a discrete molecule or it can be a very small part of complex mixture of biological substances.

PCR can be compared to biological amplification (growth in culture) with enzymatic duplication and

amplification of specific nucleic acid sequences. PCR techniques can be modified to yield results comparable to isolation of several types of microorganisms on a primary isolation medium and isolation of a single type of organism from a mixture using selective medium. The advantage of PCR over culture methods is that microorganisms that cannot be grown in culture can be detected. Shariff et al (2000) have furnished a candid account on the importance of standardization of PCR techniques in disease diagnosis.

Nested PCR

In nested PCR or 2-step PCR (as against conventional 1-step PCR), the larger fragment produced by the first round of PCR is used as the template for the second round of amplification. The sensitivity and specificity of both DNA and RNA amplification can be dramatically increased by using nested PCR method. Two step PCR was essential to detect WSSV (white spot syndrome virus) in clinically asymptomatic shrimps and in other carrier animals. Only 5% apparently healthy post larvae gave positive reaction in the conventional 1-step PCR where as 48% showed positive reaction in 2-step PCR (Otta et al., 1999 as quoted by Karunasagar, 2000). Nested PCR can reach 1000 times more sensitive than the 1-step PCR (Shariff et al., 2000).

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, and great care must be taken when performing such PCRs, particularly in a diagnostic laboratory. In fact, one molecule of a contaminating PCR template in the first step reaction may be sufficient to obtain a false positive result by nested PCR. The nested PCR is also more costly since two steps are involved and cost can increase dramatically if assays are repeated when contamination occurs.

RT PCR

Amplification of RNA by PCR can be performed by annealing a primer to the RNA template and then synthesizing a cDNA copy using reverse transcriptase (RT) (Fig. 5).

Some DNA polymerases, such as the

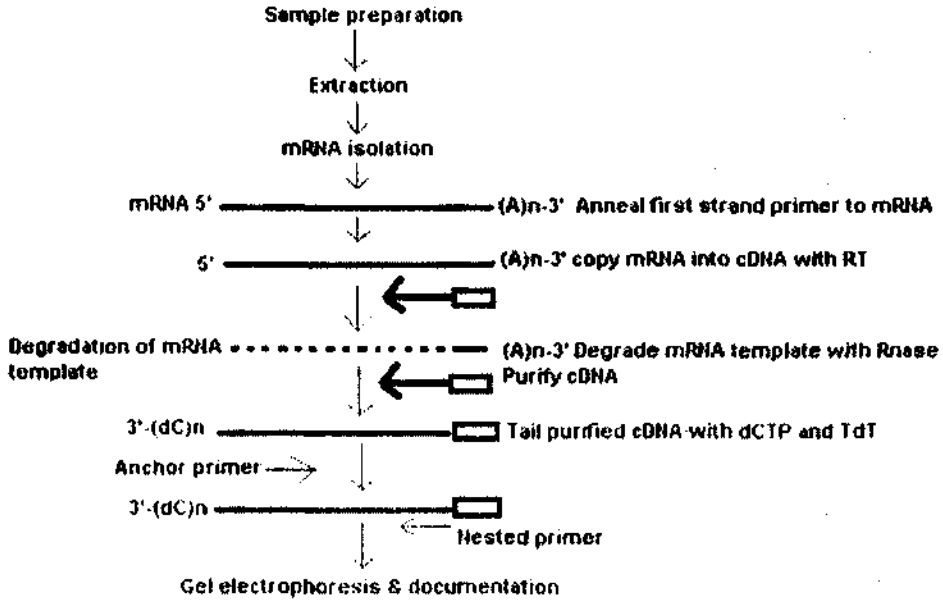

Fig.5 Schematic of the procedure of RT PCR

thermostable T.*thermo-phylus* (*Tth*), in the presence of manganese, can reverse transcribe RNA. Since *Tth* DNA polymerase can utilize both DNA and RNA, the whole procedure can be carried out in a single tube.

For mRNA that contains a poly(A) tract at the 3' end, oligo dT, random hexamers or a gene-specific primer can be used to prime cDNA synthesis. Viral RNA templates (e.g. retroviruses, rhinoviruses, etc.) or poly(A) RNA can be copied using random hexamers or specific target primers.

RNA (RT) PCR is a highly sensitive tool in the study of gene expression at the RNA level and, in particular, in the quantification of mRNA or viral RNA levels. RNA PCR can also be used as a first step in preparing a cDNA library by PCR of all of the mRNAs in a sample of cellular RNA. Genmoto et al. (1996) described a 16S rRNA targeted RT PCR for the detection of *Vibrio penaecida* from *Penaeus japonicus*.

Quantitative PCR

The exponential nature of PCR amplification lends it intrinsically to quantitative analysis. However, anything that may interfere with the exponential amplification will introduce errors. Therefore, for quantitative amplifications the PCR protocols should be tailored to minimize factors capable of affecting exponential amplification. Also in this context, PCR is not wholly exponential since the reaction plateaus at around 10^8 copies of an amplicon. Therefore for quantitative work, PCRs should be maintained within about 20 cycles, during which amplification is linear.

Number of starting molecules is critical in deciding the number of cycles to be chosen. One study has shown that the reaction is linear up to 30 cycles with 12-400 starting copies, up to 25 cycles with 200-3200 starting copies and up to 20 cycles with 3200-51200 starting copies (Kellog et al., 1990). Ideally, the linear range of amplification for a given sample type should be defined as a prerequisite to quantitative PCR. This can be achieved using controls or standards. Standards would be close to target sequences, having similar amplification kinetics and remain in the linear PCR range. For some target sequences standards are available commercially, known as mimics.

PCRs carried out using DNA or cDNA as substrate can be spiked with varying amounts of mimic target which is amplified using the same PCR primers as the real target sequence. After gel electrophoresis the relative intensities of the target and mimic PCR products are compared. The

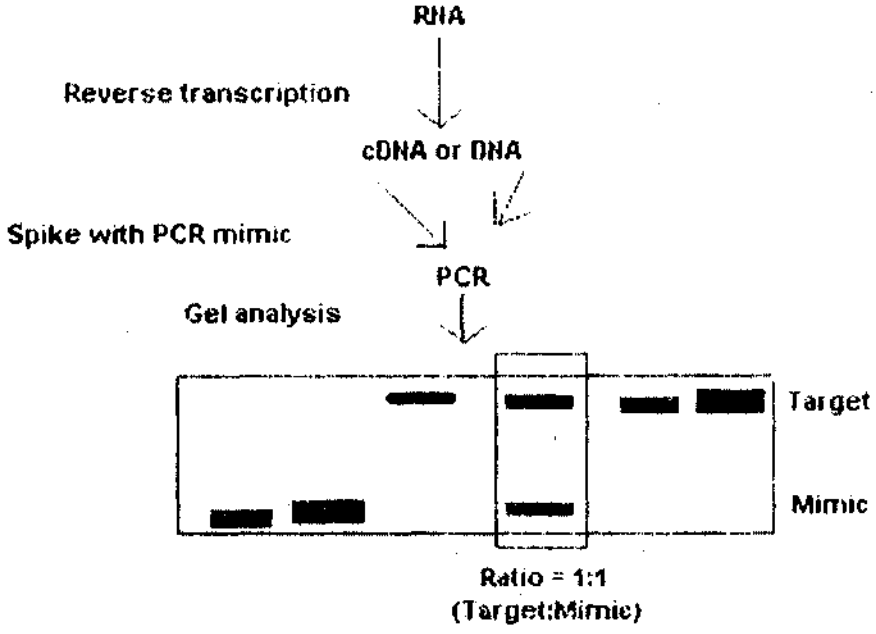

Fig.6 Quantitative PCR using standards (PCR mimics)

concentration of the target is determined by extrapolation from the PCR mimic products (Fig. 6).

The amplified target sequence is detected and quantified using labeling – either radioactive labeling using ^{32}P-end labeled primers or using non radioactive methods followed by detection with autoradiography or phosporimaging.

Remarks

Molecular techniques might serve well as confirmatory screening to reinforce/refute results from general screening methods both for establishing zones and for certifying stocks free of specific pathogens. This would reduce the sample size and frequency required for high technology screening, making their application more practical and easy to justify. Ideally the confirmatory screening should be on the same specimen (or sub-samples) from the same collections to ensure cross-reference validity (McGladdery, 2000).

Although molecular genetic methods can provide rapid and accurate information on the infection status, there is considerable risk of misdiagnosis if the various parameters that determine reaction specificity are not carefully monitored and controlled. In each of these methods, diagnostic specificity is determined by a hybridization (annealing) reaction in which a DNA (or RNA) probe must bind the target sequence in the infecting virus. A number of parameters, including reaction conditions and degree of matching of probe with target sequence determine success of diagnosis. This is particularly a problem in PCR tests for which a single base mismatch can sometimes prevent primer efficient extension of the primer-template hybrid (Walker and Cowley, 2000).

Further, the extreme sensitivity of PCR may allow amplification of DNA from non-viable or non-pathogenic organisms. A positive result does not necessarily mean that viable, pathogenic organisms are present and that morality will eventually occur. Failure to use a negative control can lead to the interpretation of negative virus infection detection in a test, which was actually a failed PCR! More research and experience is needed to fully under stand the meaning of PCR-positive results for aquaculture and management. Nonetheless, molecular diagnostic techniques hold great promise for the early diagnosis of disease outbreaks in aquaculture and natural populations.

References

Brown, T.A. 1990. *Gene cloning - An Introduction*. 2nd Edition Chapman & Hall India. 286 pp.

Genmoto, K., T. Nishizawa, T. Nakai and K. Muroga 1996. 16S rRNA targeted RT-PCR for the detection of Vibrio penaeicida, the pathogen of cultured kuruma prawn *Penaeus japonicus*. *Dis. Aquat. Org.*, 24: 185-189.

Karunasagar, I. 2000. Application of polymerase chain reaction for detection of shrimp pathogens in India. *In*: DNA-based molecular diagnostic techniques: research needs for standardization and validation of the detection of aquatic animal pathogens and diseases. Walker, P and Subasinghe, R (eds.). *FAO Fish. Rep.*, No. 395, Rome, FAO. 52-53.

Karunasagar, I. and I. Karunasagar 1998. Molecular probes and polymerase chain reaction (PCR)-based kits for diagnosis of shrimp diseases. *Naga, the ICLARM Q.*, 21 (1) Jan-Mar., 26-30.

Kellog, D.E., J.J. Sninsky and S. Kwok 1990. *Anal. Biochem.*, 189: 202.

Lightner, D.V. and R.M. Redman 1998. Shrimp diseases and current diagnostic methods. *Aquaculture*, 16: 201-220.

McGladdery, S. 2000. Technological constraints to disease prevention and control in aquatic animals, with special reference to pathogen detection. *In*: DNA-based molecular diagnostic techniques: research needs for standardization and validation of the detection of aquatic animal pathogens and diseases. Walker, P and Subasinghe, R (eds.). *FAO Fish. Rep.*, No. 395, Rome, FAO. 17-24.

Old, R.W. and S.B. Primrose 1994. *Principles of Gene Manipulation – An Introduction to genetic engineering*. 5th Edition Blackwell Scientific Publications, 474 pp.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis and H.A. Erlich 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.

Shariff, M., S. Soon, K.L. Lee and L.T. Tan 2000. Practical problems with PCR detection in Asia: The importance of standardization. *In*: DNA-based molecular diagnostic techniques: research needs for standardization and validation of the detection of aquatic animal pathogens and diseases. Walker, P and Subasinghe, R (eds.). *FAO Fish. Rep.*, No. 395, Rome, FAO. 45-51.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, 98: 503-517.

Walker, P.J. and J.A. Cowley 2000. Viral genetic variation: implications for disease diagnosis and detection of shrimp pathogens. *In*: DNA-based molecular diagnostic techniques: research needs for standardization and validation of the detection of aquatic animal pathogens and diseases. Walker, P and Subasinghe, R (eds.). *FAO Fish. Rep.*, No. 395, Rome, FAO. 54-59.

Walker, P.J. and R. Subasinghe (eds) DNA-based molecular diagnostic techniques: research needs for standardization and validation of the detection of aquatic animal pathogens and diseases. *FAO Fish. Rep.*, No. 395, Rome, FAO. 2000. 93p.