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WINTER SCHOOL

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Course Manual

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PCR BASED RAPID DIAGNOSIS OF PATHOGENS

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Introduction

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

Two of the PCR assisted diagnostic techniques merits special mention. They are the DNA fingerprinting based an arbitrarily primed PCR (AP PCR) and amplification of species specific virulence gene using specific primers flanking these genes. Before going to the details of these techniques let us examine how the PCR work

The Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a technique for the *in vitro* synthesis billions of copies of a specific nucleic acid sequence by performing successive rounds of *in vitro* nucleic acid replication. This is achieved by using two oligonucleotide primers that hybridize (annealing) to the opposite strand of the target DNA at positions that flank the region to be amplified through simultaneous extension of both primers. A repetitive series of cycles involving template denaturation, 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 synthesised in one cycle can serve 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 standard PCR mixture in addition to the sample (template) DNA contains 50mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 0.23 μm of each primer, 200 μm of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP and dTTP) and 2.5 units of Taq polymerase. The sample DNA generally contains 10^2 to 10^5 copies of template. The volume is made up to 25 or 50μl. The amplification is performed in a DNA thermal cycler, each cycle consisting of denaturation at 94°C for 30- 50 sec, annealing at 55°C for 30- 90 sec and extension at 72°C for 60- 120 sec for total of 30 cycles. Cycling could include a final extension at 72°C for 5 min. Reactions are stopped by chilling at 4°C or by addition of EDTA at 10mM.

Identification of pathogens through PCR 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 are 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 bacterial pathogens, application of this method shall become more common.

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

Bacterial Species	Gene
Vibrio vulnificus	cth (Cytotoxin haemolysin gene)
Vibrio cholera	ctxA (Cholera toxin gene) ctxB
V. parahaemolyticus (K+)	tdh (Thermostable direct haemoysin gene)
Listeria monocytogene	dth (Delayed hypersensitivity factor) Iap (Invasion associated protein gene) in1A (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 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 two20 bp primers viz;

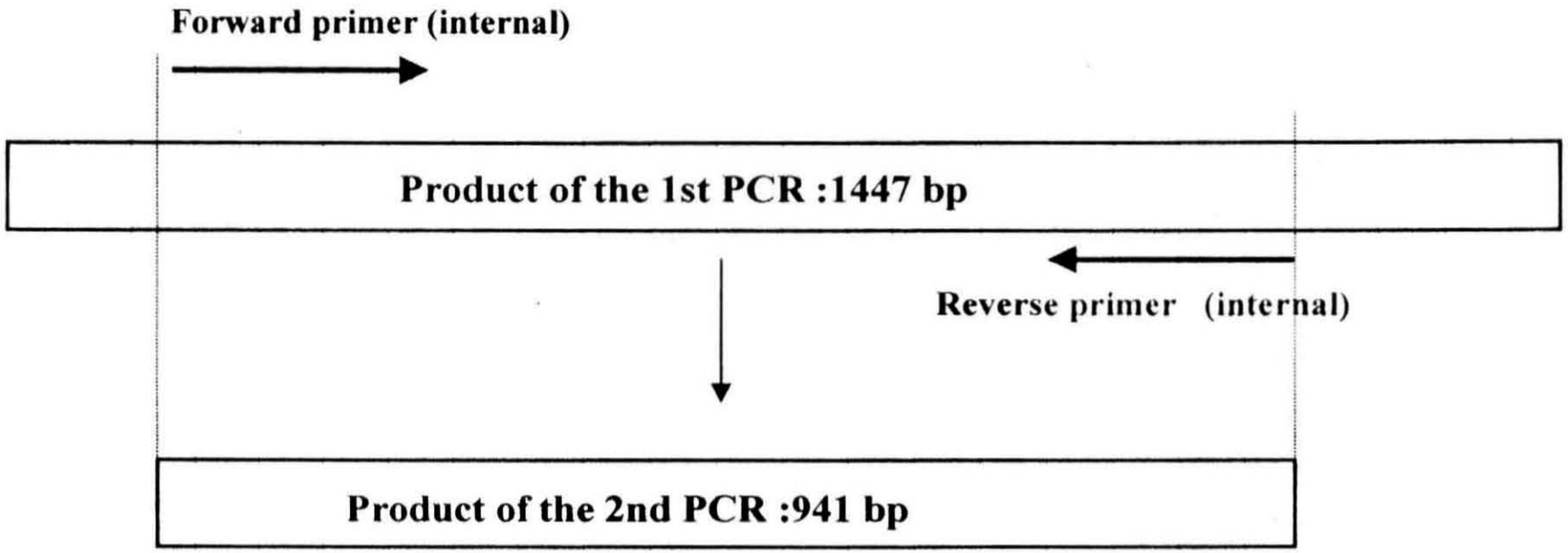
5'CTA ATC AAG ACA ATA AAA TC and

5'GTT AGT TCT ACA TCA CCT GA

was found to be useful for identifying L.monocytogenes. 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.

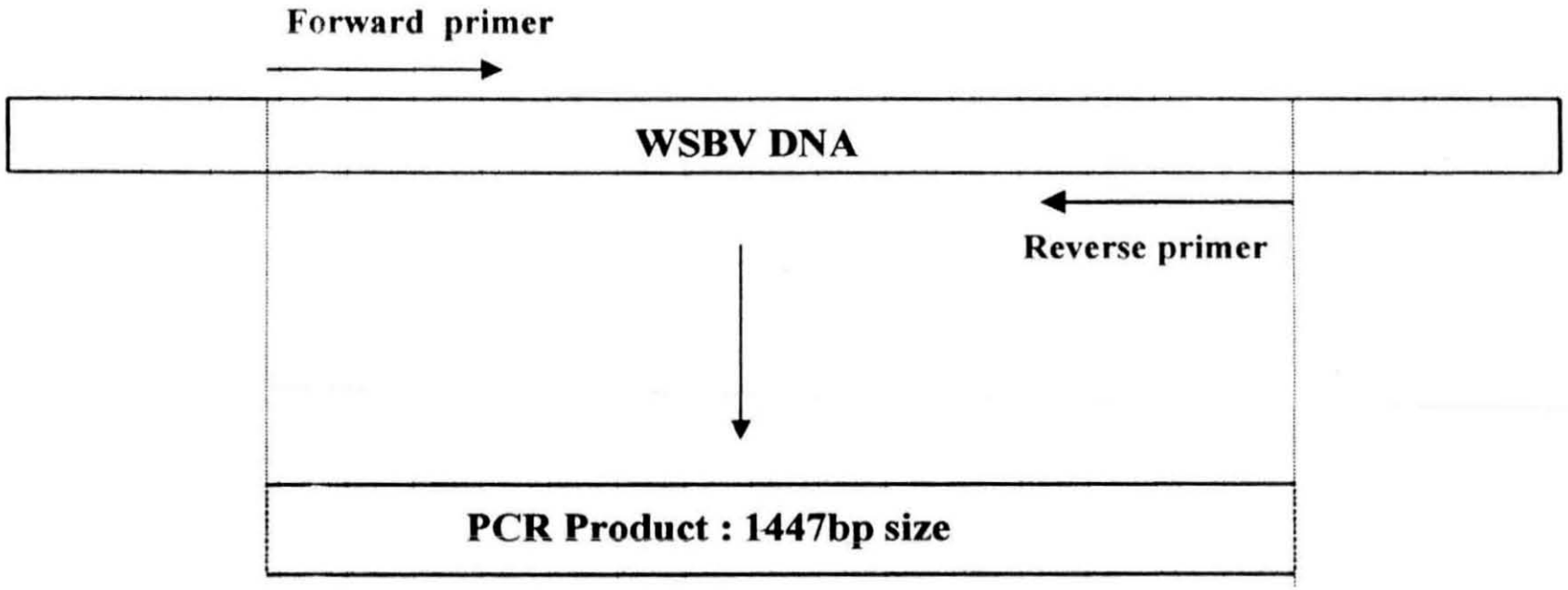
Nested PCR of W S S V

(Primers based on *Sal* I 1461bp segment of WSBV)

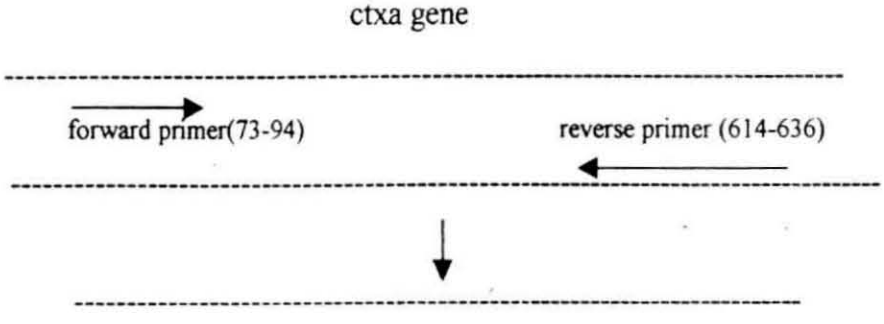


PCR amplification WSSV

(Primers based on *Sal* I 1461bp segment of WSSV)



PCR amplification of ctxA gene segment of *V. Cholerae*



(Expected PCR product) 564bp

The sensitivity and specificity of the PCR can be further enhanced by carrying out nested PCR. The process utilizes two consecutive PCRs. The first PCR utilizes a pair of primers 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. 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.

Detection of White Spot Syndrome Virus

White spot disease caused by White spot syndrome (WSSV) is one of the major threat faced by the shrimp farming industry. As there is no cure for this disease, stocking diséase free larvae is one of the management measures recommended to prevent critical transmission of the virus.

Till recently, the diagnostic technique were largely dependent upon history of disease, clinical signs and histological examination of moribund animals. From the practical point of view, these procedures are of limited use in disease prevention and sensitivity is often limited.

Of late, biotechnological diagnostic techniques using DNA probes, PCR amplification of conserved DNA sequence using specific primers are being applied to detect the presence of virus in the animal even in the latent form. These tests are highly specific and highly sensitive.

DNA probes could be used to detect the virus in clinically infected animals either using tissue extracts or through *in situ* hybridization of shrimp tissue sections. The virus can be clearly localized in the shrimp sections. Strong labeling is limited to infected nuclei and is strong only when there is hypertrophy of the nucleus. When infection is low, histological diagnosis is particularly difficult. In contrast, diagnosis with gene probes is more specific and

sensitive and it could be used as a diagnostic tool for WSBV infection using haemolymph samples from live shrimp.

PCR Screening of White Spot syndrome Virus

The infection can be either vertical transmission ie, from parent to the larvae or horizontal transmission i.e., from animal to animal. Hence, stocking of disease free larvae produced by disease free parents is of the primary requirement in the prevention and control of disease. Thus, use of very sensitive molecular diagnostic tests which can detect very low levels and latent forms is very essential. PCR amplification of single copy gene of the virus is a test which can meet these requirements. Among the various kinds of molecular diagnostic kits being marketed for White spot disease virus detection, the PCR based detection is the most sensitive and very useful for the pro-active disease management. This is much more sensitive than the nucleic acid probes. While the sensitivity of most probes is around 10^4 - 10^5 molecules of a homologous target, on an average less than 10 target molecules are sufficient to provide a positive result by PCR based test. PCR based method can be used to detect WSSV in brood stock larvae and other carrier animals. Stocking only WSSV negative larvae in culture system is the essential step in preventing vertical transmission of the disease into the cultured shrimps.

During the culture period monitor shrimp health with PCR screening. It is necessary to monitor the disease status routinely. Samples should be collected every fortnight and sent to a reliable laboratory for PCR screening. The PCR technique will detect early infection and enable the farmers to adapt a suitable strategy to minimize losses. Diagnosis relying only on the appearance of white spots does not help as by then the shrimps will die within a few days resulting in severe losses.

Duplex PCR Screening of White Spot disease Virus

CMFRI has designed a duplex PCR for detection of WSSV which is cost effective, faster and reliable compared to the nested PCR kits being used currently. This involves the simultaneous PCR screening of 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 that of a single run of the nested PCR, it is less expensive.

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

Suggested Readings

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