Molecular Probes and Polymerase Chain Reaction (PCR)-based Kits for Diagnosis of Shrimp Diseases

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Abstract

Technology for effective and fast diagnosis of animal diseases is essential for developing aquaculture management strategies. This paper reviews the conventional techniques for shrimp disease diagnosis and discusses the emergence of nuclei acid probes and polymerase chain reaction (PCR)-based kits as powerful tools for rapid and accurate detection of shrimp diseases.

Introduction

Rapid diagnosis of diseases is essential in aquaculture management, not only to facilitate control of diseases but also to help in developing strategies for management against diseases and to prevent the spread of diseases. Lack of tools to accurately diagnose disease often leads to indiscriminate use of chemicals and antibiotics resulting in environmental problems such as emergence of antibiotic resistant pathogens and antibiotic residues in the raw materials. In India, indiscriminate use of antibiotics in aquaculture has already resulted in the emergence of resistant organisms and pathogens in the environment (Karunasagar 1994; Karunasagar et al. 1994, 1996a). Rapid diagnostic methods would greatly help disease surveillance and monitoring programs, assessment of efficacy of treatment regimes and identification of sources of contamination. Rapid detection methods for pathogens would enable hatcheries and farms to screen broodstock and larvae.

Problems with Conventional Methods

Viral diseases: Viral diseases have been devastating shrimp aquaculture, as recently experienced in India. Viral diseases are presently diagnosed through epidemiological features, gross discernible structural changes, wet mount microscopy with or without staining, histopathology and electron microscopy. Specific antibody and DNA probe-based methods are available for only a few viruses. Conventional microscopic methods allow recognition of the acute phase of infections but lack sensitivity to detect latent or carrier state infections and do not allow discrimination between antigenic differences, geographic diversity in virulence, etc. Detection of reo-like viruses is problematic because the histopathological method lacks sensitivity (Brock 1992) and complications arise when cellular changes suggestive of viral infection (e.g., in the lymphoid organ) are encountered in clinically normal shrimp (Brock 1992).

In addition, shrimp cell lines are not available at present so it has not been possible to culture shrimp viruses. The only way to propagate shrimp viruses is by infecting a fresh stock of hosts with the risk that these hosts may carry other latent viruses.

Bacteria: Bacteria are associated with both endemic and epidemic diseases of shrimp and have caused large-scale mortalities. Standard microbiological methods are applied to detect and identify bacterial agents from shrimp tissues. Fortunately, the majority of bacteria associated with shrimp diseases can be easily isolated and identified by classical methods. However, these methods are time consuming and require at least 2-3 days for accurate identification. It is also essential to isolate the bacteria to determine antibiotic sensitivity profiles. Isolation methods can be tedious because some bacteria,

e.g., the filamentous bacterium *Leucothrix mucor*, and acid fast bacteria, are fastidious and require specialized media for their culture.

Fungi: Several fungi belonging to the group phycomycetes (e.g., *Lagenidium* sp., *Sirolpidium* sp. and *Haliphthoros* sp.) and *Fungi imperfecti* (e.g., *Fusarium* sp.) are important shrimp pathogens. Diagnosis of fungal infections can be established through direct microscopic examination and culture. Most of the shrimp pathogenic fungi can be isolated on standard mycological media. However, identification based on these methods is time consuming.

Protozoa: Several protozoan ectoparasites (e.g. *Zoothamnium*, *Epistylis*) and endoparasites (gregarines and microsporidians) cause problems in penaeid shrimp culture. Microscopic examination of wet mounts or histological preparations are presently used to detect these parasites.

Involvement of multiple etiological agents: Diagnosis of diseases is complicated by the fact that disease outbreaks in cultured shrimp are the result of an interaction between multiple etiological factors (Brock 1992). It is important to understand the relationship between pathogens. One of the approaches used to clarify associations between biotic agents and clinical disease is the determination of the prevalence of infection and quantitative estimates of abundance of putative pathogens. In situations where it is not possible to determine directly the number of particular pathogens, e.g., viral inclusion bodies, extent of cytological damage or haemocytic nodules, structural changes surrounding bacterial colonies are counted to derive severity estimates.

Nucleic Acid Probes for Diagnosis

Since DNA probes for detection of entero-toxigenic *Escherichia coli* were first applied by Moseley et al. (1980), there has been a growing interest in using this technique in the diagnosis of infectious diseases. DNA probes capable of identifying a number of bacterial, viral, fungal, parasitic and helminthic pathogens are currently being used in medical diagnostic laboratories.

Nucleic acid probes are segments of DNA or RNA that have been labeled with enzymes, antigenic substrates and chemiluminescent moieties or radioisotopes. They can bind with high specificity to complementary sequences of nucleic acid. Probes can be directed to either DNA or RNA targets and can be twenty to thousands of bases long. Oligonucleotide probes (generally less than 50 base pairs (bp)) 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 recent years non-radioactive probes have become increasingly popular. Commercial kits for labeling probes with affinity labels such as biotin and digoxigenin are available. Moreover, chemiluminescent markers have sensitivity ranges equal to or greater than ^{32}P labeled probes. They also have longer shelf lives and do not necessitate the additional safety and control measures required by the use of ^{32}P.

Nucleic acid hybridizations can be performed in solutions or on solid supports, e.g., filters. Though the sensitivity of the latter is less than the former, it facilitates handling multiple samples. *In situ* hybridization assay, in which whole cells or tissue sections fixed on slides are taken through the hybridization process, is a variant of solid phase hybridization assay and has become an important diagnostic tool.

Nucleic acid hybridization tests are extremely useful for detection of pathogens for which traditional culture techniques are not available. Even with organisms which can be cultured, nucleic acid hybridization tests decrease the time necessary to identify fastidious microorganisms and allow differentiation of pathogenic and non-pathogenic strains which may be biochemically similar. By targeting virulence-associated genes, the virulent strains can be differentiated from avirulent ones. In shrimp disease diagnostics, nucleic acid probes have great potential for diagnosis of viral disease. Lightner et al. (1994) described development of a DNA probe for infectious hypodermal and hematopoietic necrosis virus (IHHNV). It was noted that the probe recognized IHHNV infected tissues that were not detected as readily in haematoxylin and eosin stained sections. DNA probes are presently available (Lightner 1996) to detect several penaeid shrimp viruses including IHHNV, hepatopancreatic parvo-like virus (HPV), monodon baculovirus (MBV), baculovirus penaei (BP), white spot baculovirus and taura syndrome virus (TSV). *In situ* hybridization techniques using histological sections have the advantage that non-specific tissue effects, which may lead to false positive reactions in dot blot assay, can be distinguished from

specific histological lesions which react with labeled probe (Lightner 1996). Most of the available literature relates to the development of probes and their application in experimentally infected animals. There are few reports of application of these methods to naturally infected shrimp and the sensitivity of these methods in the field.

PCR-based Tests

Polymerase chain reaction (PCR) is an *in vitro* method for enzymatic amplification (through repeated duplication) of a specific segment of DNA. Although the basic theory for the *in vitro* nucleic acid amplification method (in which there is extensive synthesis of a tRNA gene by primer directed DNA synthesis) was postulated in 1971 (Kleppe et al.), it did not result in an exponential amplification process which is the hallmark of PCR. The concept of PCR was developed by Kary Mullis, R.K. Saiki and co-workers during the 1980s (Saiki et al. 1988). The development of nucleotide sequencing methods, the storage of this information in a computer researchable database, the invention of automated oligonucleotide synthesis methods, and the discovery of thermostable DNA polymerase, have all contributed to the rapid implementation and widespread use of PCR today.

PCR involves a series of DNA denaturation, annealing and synthesis in the presence of oligonucleotide deoxytriphosphates and a thermostable DNA polymerase under specific temperature conditions to replicate specific DNA segments (Fig. 1). This series of steps is repeated for a number of cycles thereby producing up to a million copies of DNA fragments.

There are several ways in which PCR fragments can be characterized. A common method is to determine the size of the amplified fragments by using gel electrophoresis. The amplified segment can also be sequenced. This is laborious and is not suited for routine analysis of large numbers of samples, but provides the strongest confirmatory data. Since the sequence of the amplified region is generally known, conveniently located internal restriction endonuclease clear cleavage sites can be located and the size of the fragments can be predicted. This can be confirmed by agarose gel electrophoresis of cleavage products and by comparing the size of the observed fragments with the predicted size. An alternative approach is to use an internal hybridization probe on a Southern blot of the gel.

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

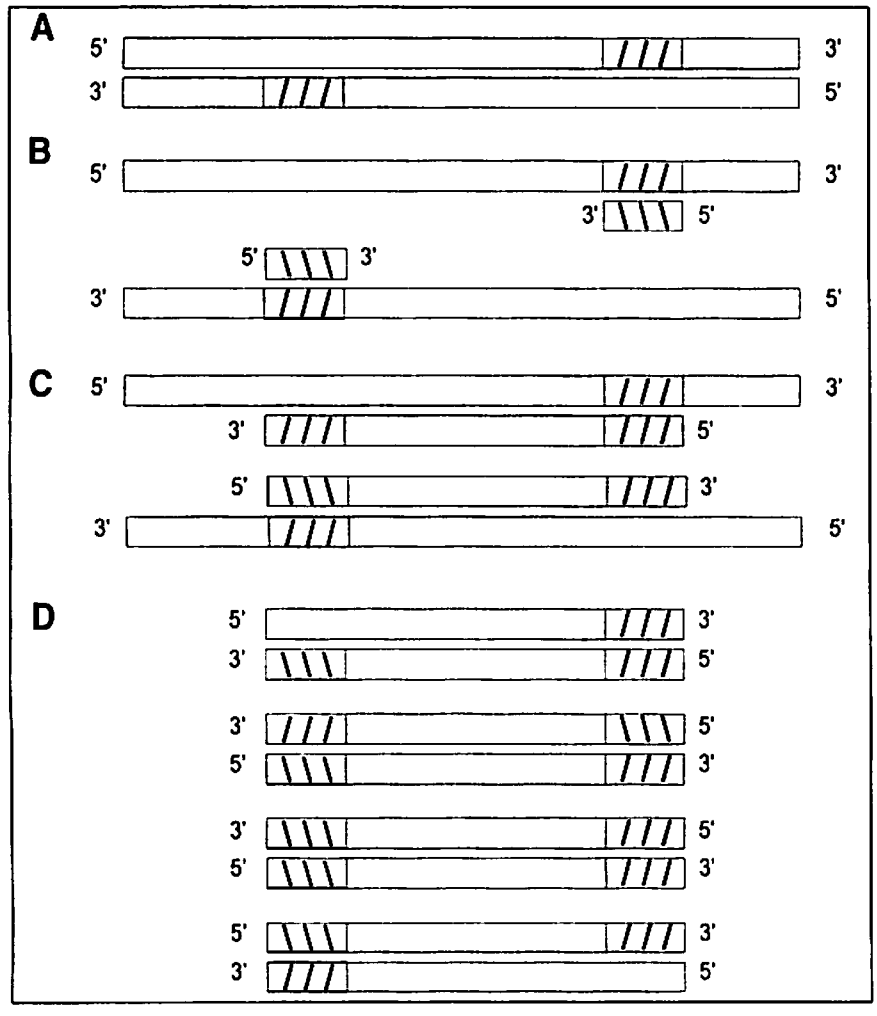

Fig. 1. Basic polymerase chain reaction.

(A) The primer binding sites on the template. The region of DNA to be amplified (the target DNA) is defined by a pair of oligonucleotide primers. The 3' end serves as initiation point for DNA replication.

(B) The DNA strands separated by heat (94°C) and on cooling to annealing temperature, the primers bind to the template producing a target DNA-primer hybrid complex.

(C) New strands are synthesized by Taq polymerase (a thermally stable DNA polymerase) on the template.

(D) In the next cycle, 8 fragments with defined ends are synthesized from 4 templates of the previous cycle. The thermocycling is repeated usually for 30-40 cycles. Million fold increases in DNA are common.

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 using PCR.

PCR has advantages over other methods that use nucleic acid probes. The sensitivity of most probes is around 10^4 to 10^5 molecules of a homologous target. This is not sufficiently high for them to be used for direct detection in clinical specimens. By PCR, on an average, fewer than 10 target molecules are sufficient to provide a positive result. The sensitivity can be further increased by targeting molecules which are present in multiple copies in a single cell e.g., rRNA sequence.

Discussion

There is tremendous interest in the use of PCR technology for diagnostic purposes. Several pathogenic microorganisms can be detected directly from clinical samples using PCR methods (Persing et al. 1994). In our laboratory, we have developed methods to detect pathogenic *Vibrio* sp. directly from fish and shrimp (Karunasagar et al. 1995, 1996b, 1997). The technique for detection of *V. cholerae* has a sensitivity of 10^3 cells when PCR is performed directly from shrimp and less than 10 cells if PCR is preceded by 6 h enrichment in alkaline peptone water. Another advantage of the method is that it can detect the newly identified toxigenic serotype *V. cholerae* 0139 which may be missed in conventional methodology using 01 antiserum (Karunasagar et al. 1995). In the case of *V. parahaemolyticus*, the human pathogenic strains produce a thermostable direct haemolysin encoded by *tdh* gene, and by targeting this gene, it is possible to detect pathogenic strains in seafoods by PCR. Direct detection from fish samples has a sensitivity of 10^4 cells, whereas 10 h enrichment can increase the sensitivity to less than 10 cells (Karunasagar et al. 1996b).

V. parahaemolyticus is also an important shrimp pathogen irrespective of haemolytic (Kanagawa) activity, and it is important to detect the entire *V. parahaemolyticus* population. For this purpose a PCR which targets a chromosomal region of unknown function has been found useful (Lee et al. 1995). PCR technique has been found to be applicable for detection of *V. parahaemolyticus* strains directly from infected shrimp samples (Karunasagar et al. 1997). Hill et al. (1991) described PCR amplification of the *V. vulnificus* specific cytolysin gene as it is an important shrimp pathogen. Genmoto et al. (1996) described a 16S rRNA targeted reverse transcription PCR for detection of *Vibrio penaecida*, a pathogen of the cultured Kuruma prawn.

PCR technology has the potential to be one of the most useful tools for the diagnosis of viral diseases of shrimp. Many scientists have already used the techniques or have indicated their advantages in a variety of applications. Lee et al. (1993) used PCR techniques to amplify a fragment of MBV polyhedrin gene which could be used as a specific probe in hybridization assays. Lightner (1996) noted that PCR could be a useful non-lethal method for detection of IHHNV in broodstock. Lightner et al. (1994) indicated that PCR methods for IHHNV matched or exceeded *in situ* hybridization in diagnostic sensitivity. Lo et al. (1996) described a set of PCR primers for detection of white spot baculovirus (WSBV) in penaeid shrimp. Takahashi et al. (1996) noted that the PCR primers designed for rod shaped nuclear virus of *Penaeus japonicus* (RV-PJ) reacted with WSBV indicating that these two viruses are closely related. Wang et al. (1996) described PCR-based detection of BP in postlarvae. They emphasized that the procedures for preparing samples for PCR are critical because unknown compounds in shrimp tissue inhibited DNA polymerase.

More field data on the application of PCR-based methods for naturally infected or carrier shrimps are necessary to assess their importance as screening tests for broodstock, larvae and juveniles in aquaculture. PCR-based molecular typing methods such as Random Amplification of Polymorphic DNA (RAPD) are emerging as powerful tools in epidemiology. PCR-based typing methods also have an immense potential for application in the field of fish and shrimp epizootiology.

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