Diagnosis, treatment and prevention of microbial diseases of fish and shellfish

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Microbial diseases are a major problem for aquaculture all over the world. The problems faced by shrimp aquaculture industry in India is a classic example. The potential area available for brackish water aquaculture in India has been estimated to be 1.19 million ha, of which only 0.18 million ha has been brought under culture[1] till 1995–96. Cultured shrimp contributed to only 20.3% of total export of 49,000 tons during 1986–87, but the contribution increased to 48.7% of 90,000 tons[1] during 1995–96. During 1994–95, disease problem struck the booming aquaculture industry and caused a loss of 10,000–12,000 tons valued at Rs 250–350 billion[2]. Disease management strategies are dependent on rapid detection of a problem before it reaches epizootic proportions. The purpose of this review is to present the current status of disease diagnosis methods, treatment options and potentials for immunoprophylaxis in aquaculture systems.

Disease diagnosis

A variety of microbial agents cause disease problems in aquaculture systems. These include viruses, bacteria, fungi and parasites. Diagnosis of disease is generally established by clinical signs and demonstration of the pathogen in the lesions or organs. Fish/shrimp pathogens are generally of two types: Primary pathogens which cause serious problems to animals even in low stress environment and opportunistic pathogens which normally exist within the natural environment of the fish/shellfish and are only capable of attacking a host whose normal defences are compromised by stress. Diagnostic methods should be able to differentiate pathogenic organism from the commensal flora. Details of methodologies used for sampling, clinical examination, histopathology and bacteriology have been presented in some excellent books[3–5]. While clinical signs and histopathological changes provide useful lead for diagnosis, accurate diagnosis can be established by isolating and identifying pathogens. However, isolation may not be possible in some cases, e.g. shrimp viruses due to non-availability of cell culture systems and difficulties in culturing parasites. For effective health management in aquaculture systems, it would be important to detect subclinical stages of infection and also identify carriers of pathogens. Conventional methods of diagnosis based on histopathology cannot achieve these. However, antibody-based and nucleic acid-based methods offer solutions to these problems and further discussions would be limited to these methods.

Nucleic acid-based methods for disease diagnosis

Since the first application of DNA probes for detection of enterotoxigenic *Escherichia coli*[6], there has been a growing interest in using this technique for diagnosis of infectious diseases. Nucleic acid probes are segments of DNA or RNA that have been labelled with enzymes, antigenic substrates, chemiluminiscent moieties or radioisotopes and can bind with high specificity to complementary sequences of nucleic acid[7]. Probes varying in length from 20 to thousands of base pairs can be used and may be directed towards DNA or RNA. Presently nucleic acid hybridization tests are widely used in medical and veterinary diagnostic fields.

There are a number of reports of application of nucleic acid probes for diagnosis of fish and shrimp diseases (Tables 1 and 2). Rimstad *et al.*[8] developed an oligonucleotide probe homologous to a part of the nucleotide sequence coding for a protease of infectious pancreatic necrosis virus (IPNV) affecting Atlantic salmon. The DNA–RNA hybridization assay could detect several field strains of IPNV and was not serotype-specific. Hence this assay would be a useful alternative to virus isolation in cell cultures or neutralization assay[8]. Similar strategy was used for development of cDNA probe for the RNA virus, aquareovirus[9]. Among fish pathogenic bacteria, *Renibacterium salmoninarum* appears to have attracted most attention. This may perhaps be due to the difficulties in diagnosis of bacterial kidney disease by conventional methods. This bacterium is rather slow growing and takes 7–15 days to grow in culture media[10]. Mattson *et al.*[11] developed an oligonucleotide probe hybridizing with the 16S rRNA of *R. salmoninarum*. This probe was found to be specific for this organism and in filter hybridization assays, the detection limit was 2.5×10^4 bacteria for clinical samples. A biotinylated DNA probe for the same organism has been developed[12] and the

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sensitivity of dot blot hybridization assay was shown to be similar to that of culture.

Nucleic acid hybridization methods have been found to be extremely useful for shrimp pathogens, particularly viruses, which are difficult to isolate and purify due to non-availability of shrimp cell lines. Mari *et al.*[13] developed gene probe for infectious hypodermal and haematopoietic necrosis virus (IHHNV) affecting shrimp. The viral DNA was cloned and the selected clones characterized by restriction digestion. Probe developed from one of the fragments was specific to IHHNV and did not hybridize with insect parvovirus, but hybridized with areas corresponding to intranuclear Cowdry type A inclusion bodies. Bruce *et al.*[14] used a similar approach for developing probe for *Baculovirus penaei*. With the DNA probes for monodon baculovirus (MBV), Lightner *et al.*[15] noted that the spherical occlusion bodies themselves did not react with the probe, though intense deposition of precipitate was noted in cells containing the occlusion bodies and around the outer surface of some of the occlusion bodies. This has been attributed to inadequate penetration of protective protein matrix by the probe. It was further noted that unassembled viral DNA contained in the cell nuclei was far more accessible to binding by the probes than the DNA contained within fully formed virions or within occluded virions.

The gene probe developed for hepatopancreatic parvovirus (HPV) isolated from *P. chinensis* reacted with all HPV-infected *P. chinensis* specimens. However, the probe did not react with three species of penaeid shrimp from Indo-pacific which had HPV-type intranuclear inclusions[15], suggesting genome variations in HPV strains. Durand *et al.*[16] and Lo *et al.*[17] used DNA probe to study tissue specificity of white spot syndrome baculovirus (WSSV).

Non-radioactive labelling of gene probes has made this technology easily applicable for laboratories without sophisticated facilities. The first non-radioactive gene probe for shrimp pathogens was developed using digoxygenin-11-dUTP (DIG) as the DNA label and enzyme-linked immunosorbant analysis (ELISA) based system for final detection[13]. Now field level diagnostic kits for IHHNV and WSSV based on gene probes are commercially available. These are adapted to 'dot blot' formats where homogenized shrimp tissues are blotted and fixed on nitrocellulose or nylon membranes and hybridized with DIG-labelled probes and the binding of the probe is detected using antidigoxygenin antibody with enzyme label. Similar technology has been used to develop gene probe-based kits for rickettsial type

Table 1. Reported application of nucleic acid-based methods for detection of fish pathogens

Pathogen	Method used	Ref.
Viruses		
Infectious pancreatic necrosis virus (IPNV)	Hybridization with oligonucleotide probe	8
Aquareovirus	cDNA probe	9
IPNV	Detection of viral genomic RNA in silver stain	169
Birnavirus	PCR	26
Trout haemorrhagic septicaemia rhabdovirus	PCR	25
Viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV)	Reverse transcriptase-dependent PCR (RT–PCR)	23
Birnavirus and IPNV	RT–PCR	24
Walleye dermal sarcoma virus	RT–PCR	27
Red seabream iridovirus	PCR	28
Fish iridovirus	PCR	29
Bacteria		
Renibacterium salmoninarum	Oligonucleotide probe complementary to 16S rRNA	11
R. salmoninarum	PCR	21, 170
R. salmoninarum	Biotinylated DNA probe	12
Fish mycobacteria	PCR	22
Piscirickettsia salmonis	PCR	171

Table 2. Reported application of nucleic acid-based methods for detection of shrimp pathogens

Pathogen	Method used	Ref.
Bacteria		
Vibrio penaeicida	16S rRNA RT–PCR	33
Vibrio parahaemolyticus	PCR	35
Necrotizing hepatopancreatitis (NHP) bacterium	Gene probe	18
Parasite		
Agmasoma spp.	Gene probe	37
Viruses		
Penaeus monodon-type baculovirus (MBV)	PCR, gene probe	30, 88
Infectious hypodermal haematopoietic necrosis virus (IHHNV)	Gene probe	13
Baculovirus penaei (BP)	Gene probe	14
Haepatopancreatic parvo virus (HPV)	Gene probe	15
Baculovirus midgut gland necrosis virus	Gene probe	172
White spot syndrome baculovirus (WSSV)	PCR Gene probe	31, 17 173, 16, 32, 174
Yellow-head virus	PCR	175

bacterial agent causing necrotizing hepatopancreatitis in shrimp[18] and for the microsporidian *Agmasoma* sp. which parasitizes *P. monodon* and *P. merguiensis* in south east Asia[19].

During recent years, polymerase chain reaction (PCR) has gained wide popularity as a diagnostic tool. PCR is an *in vitro* method for selective, repeated duplication of a specific segment of DNA. Though the basic ingredients for *in vitro* nucleic acid amplification method were described in 1971, in which the extensive synthesis of a tRNA gene by primer directed DNA synthesis was postulated[20], this work did not result in an exponential amplification process which is the hallmark of PCR. The concept of PCR was developed by Kary Mullis and Saiki and co-workers during the early eighties. The development of nucleotide sequencing methods, the storage of this information in a computer researchable database, invention of automated oligonucleotide synthesis methods and the discovery of thermostable DNA polymerase have all contributed to the rapid implementation and widespread use that PCR enjoys today. In PCR, the region of DNA to be amplified (the target DNA) is defined by a pair of oligonucleotide primers, the 3′ end of which serves as initiation point for DNA replication. In the first phase, DNA strands are separated (denatured) and then cooled in the presence of a billion-fold excess of primers, the four dNTPs and a thermally stable DNA polymerase. When strands are cooled, target DNA–primer hybrid complex will be formed, DNA replication will be initiated and at the end of the extension cycle, the target region will consist of one original strand and a new strand that has a defined 5′ end. The temperature of the annealing step is critical in controlling the accuracy (stringency) of the amplification process. Too low an annealing temperature will result in non-specific amplification and an array of PCR products. When the annealing temperature is too high, primers will not be stably associated with target DNA and no amplification will occur. In the second cycle, the temperature is raised again to separate double strands, then cooled to allow primer binding and DNA replication. The template strands synthesized in the first round of replication have one defined end and when they serve as template, the resulting new strand will have two defined ends and therefore, a uniform length. This length can be predicted and is the distance between the 5′ ends of each primer. The thermocycling is repeated usually for 30–40 cycles and million fold increases in DNA are common.

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 many samples but this 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 sequences. PCR techniques can be modified to yield results comparable to isolation of several types of micro-organisms on primary isolation medium and isolating a single type of organism from a mixture using selective medium. The advantage of PCR over culture methods is that certain types of micro-organisms cannot be grown in culture but such micro-organisms can be detected using PCR.

PCR has advantages over methods using nucleic acid probes. The sensitivity of most probes is around 10^4–10^5 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 less than 10 target molecules are sufficient to provide a positive result. The sensitivity can be further increased by targetting molecules which are present in multiple copies in a single cell, e.g. rRNA sequences.

There are a number of reports of application of PCR-based methods for diagnosis of fish and shrimp diseases. Leon *et al.*[21] developed PCR-based assay for the fish pathogen *R. salmoninarum* targetting a 149 base pair DNA sequence unique to this organism. The assay could specifically detect *R. salmoninarum* in kidney tissue of infected fishes. Using the conserved area of mycobacterial 16S rRNA gene, Talaat *et al.*[22] developed PCR assay for fish mycobacteria. Their assay amplified a 924 bp fragment of 16S rRNA gene and the digestion of the product with restriction enzymes *BamI* and *ApaI* gave a pattern which could differentiate *M. marinum*, *M. fortuitum* and *M. chelonae*.

Reverse-transcriptase (RT)-based PCR would be useful for detection of RNA viruses. Bruchhof *et al.*[23] developed RT-PCR for viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV) using primer pairs designed for the amplification of glycoprotein G-specific gene fragments of the two viruses. This technique was found to be highly specific and sensitive allowing differential diagnosis of VHS and IHNV within 8 h. Using RT-PCR, Rodriguez *et al.*[24] demonstrated co-existence of infectious pancreatic necrosis virus (IPNV) and a rhabdovirus in rainbow trout. The sensitivity of PCR assays can be enhanced by capturing the target organisms using antibodies. Estepa *et al.*[25] detected trout haemorrhagic septicaemia

rhabdovirus by capture with monoclonal antibodies and amplification with PCR. Novoa *et al.*[26] noted the superiority of PCR over the immunological techniques such as immunodot assay and immunofluorescent assay for diagnosing birnavirus infection in turbot. Poulet *et al.*[27] used PCR and RT-PCR for analysis of infection and transcriptional activity of walleye dermal sarcoma virus (WDSV) in the organs of adult walleyes (*Stizostedion vitreum*). Quantitative PCR was performed to estimate the number of viral DNA and RNA copies. Their data suggest that tumour-bearing walleyes harboured a transcriptionally active WDSV, while tumour-free walleyes contained mostly silent WDSV DNA.

PCR can be used to detect uncharacterized pathogens. Oshima *et al.*[28] developed a method for DNA viruses based on the widespread presence and strong conservation of the ribonucleotide reductase gene among these viruses. Using this assay, they could detect fish iridoviruses. On the other hand, Tamai *et al.*[29] designed a PCR-based on nucleotide sequence of frog virus 3 and silkworm iridiscent virus type 6. Using this method, four different types of iridoviruses could be detected in diseased fish.

One of the earliest reports of the application of PCR for detection of shrimp pathogens is for *P. monodon*-type baculovirus[30]. Lu *et al.* designed primers based on the sequence of the conserved regions of the polyhedrin gene of *Autographa californica* nuclear polyhedrosis virus (ACNPV). Lightner *et al.*[15] developed PCR for detection of IHHNV based on DNA sequence information obtained from one of their cloned DNA fragments. Takahashi *et al.*[31] and Lo *et al.*[17] used similar approach for development of PCR-based method for detection of WSSV. PCR method has been used to detect WSSV in brood stock and carrier animals[32]. In the case of WSSV, Lo *et al.*[32] noted that two step amplification using nested primers increased the sensitivity of detection by 10^3 to 10^4 times.

PCR-based methods have also been used for other shrimp pathogens such as bacteria and parasites. Genmoto *et al.*[33] reported detection of *Vibrio penaeicida* based on reverse transcriptase (RT)-PCR targetting the 16S rRNA. They noted that this method was 100 times more sensitive compared to 16S rDNA targetted PCR. Detection of *V. parahaemolyticus* in fish and shrimp by PCR targetting a chromosomal region specific to this species has been reported[34,35]. PCR-based detection of bacterium causing necrotizing hepatopancreatitis (NHP) of cultured *P. vannamei* has been achieved using nucleotide sequence of 16S rRNA gene[36].

PCR-based techniques have also been widely used in epidemiological studies to understand the relationship between pathogens. Studies on restriction fragment length polymorphism of PCR amplified products, random amplification of polymorphic DNA (RAPD) or sequencing of PCR amplified products are some of the techniques used (Figure 1). For example, Pasharawipas *et al.*[37] amplified the small subunit RNA (SSU rRNA) gene of the microsporidian parasite *Agmasoma* spp. affecting *P. merguiensis* and *P. monodon*. Degenerate oligonucleotide primers for PCR were designed based on nucleotide sequence of SSU rRNA gene of microsporidian *Vairimorpha necatrix* and *E. coli*. Amplified fragments were cloned and sequenced and the data suggest that a single microsporidian parasite affects both shrimp species. Pizzutto and Hirst[38] used M13 DNA fingerprinting to study isolates of *Vibrio harveyi* from *P. monodon* larvae.

Antibody-based methods for diagnosis of fish diseases

Antibodies, both monoclonal and polyclonal have been widely used for diagnosis of fish diseases (Table 3). Several antibody-based methods have been used including slide agglutination, co-agglutination/latex agglutination, immunodiffusion, direct and indirect fluorescent antibody tests (FAT and IFAT), immunohistochemistry (IHC), enzyme-linked immunosorbant assay (ELISA) and dot blot/dipstick and Western blot (WB). Serological tests were initially based on polyclonal antibodies (PAbs) which tend to be very convenient for use in simple tests such as slide and latex agglutination. However, cross reaction with related organisms is a problem with PAbs and often, they have to be purified before use. Pabs have been successfully used to detect carrier fish

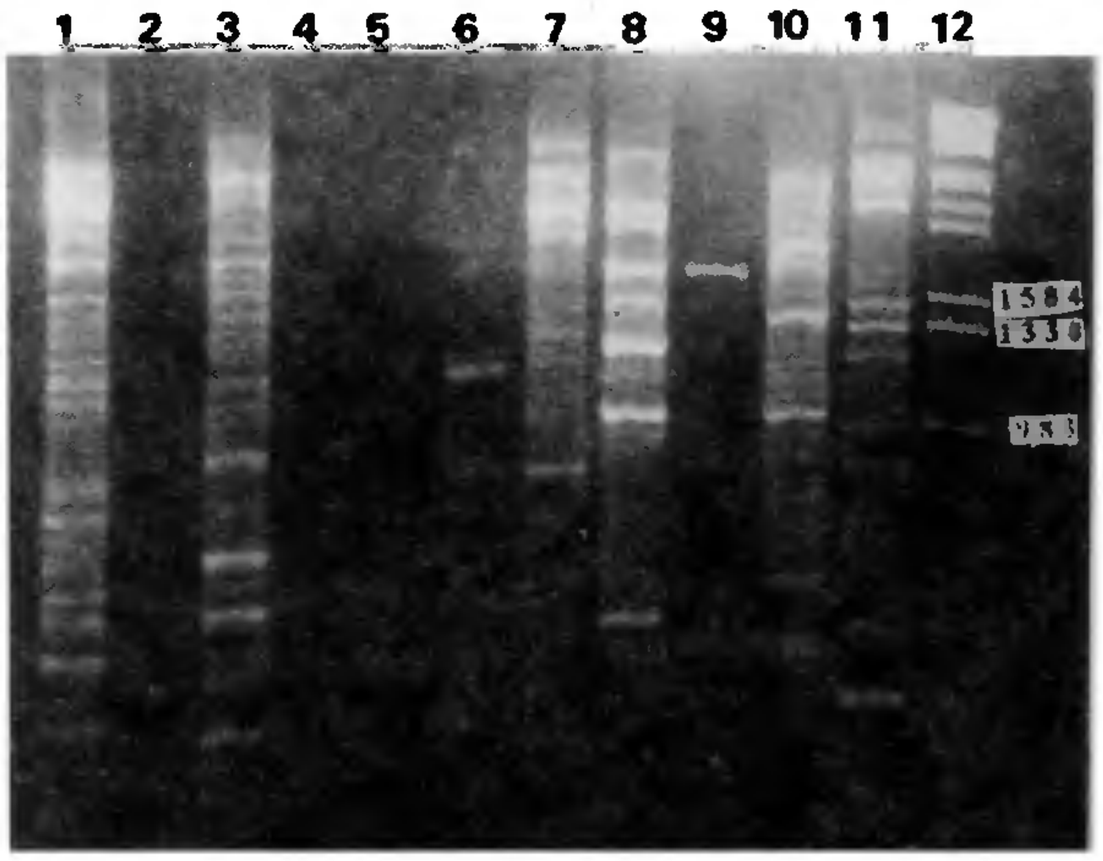

Figure 1. Application of random amplification of polymorphic DNA (RAPD) for typing of fish pathogen *Aeromonas hydrophila*. Strains of *Aeromonas hydrophila* isolated from cases of epizootic ulcerative syndrome were typed by RAPD using a single primer 5′-GCGATCCCCA-3′. Lanes 1–11, various strains of *Aeromonas hydrophila*. Lane 12 molecular weight marker. Reaction condition: initial delay 5 min and 35 cycles of 94°C 1 min, 36°C 1 min, 72°C 2 min, final delay 5 min. Karunasagar *et al.*, unpublished results.

for *Aeromonas salmonicida*[39] and in ELISAs, a sensitivity of around 10^3–10^4 bacteria per ml has been reported[40,41]. Monoclonal antibody (MAb)-based methods achieve greater sensitivity and specificity than PAbs. A combination of MAbs and PAbs in the same test may also prove useful[42].

Methods such as agglutination and coagglutination are simple to perform and do not need expensive lab equipments. Agglutination tests have been used for detection of *A. salmonicida*[43], *A. hydrophila*[44], *R. salmoninarum*[45] and *Vibrio* spp.[46]. Coagglutination has been found to be useful for detection of *A. salmonicida*[43,47,48], *R. salmoninarum*[49] and *Y. ruckeri*[50].

Table 3. Application of antibody-based methods for detection of fish pathogens

Pathogen	Method	Ref.
Aeromonas salmonicida	Agglutination	43
	Coagglutination	47, 48, 176
	Fluorescent antibody test (FAT)	55
	Immunohistochemistry (IHC)	55
	ELISA	39, 40, 68, 69
Aeromonas hydrophila	Agglutination	44
	FAT	54
Renibacterium salmoninarum	Agglutination	45
	Immunodiffusion	51, 52
	Coagglutination	49
	FAT	56–58, 177, 178
	IHC	62
	Dot blot	179, 180
	ELISA	73–75, 141
	Western blot	80–82
Yersinia ruckeri	Latex agglutination	50
	FAT	159
	IHC	181
	ELISA	69, 76
Vibrio spp.	Agglutination	46
	IHC	64
	ELISA	77, 78, 182
Edwardsiella spp.	ELISA	70, 71
Mycobacterium spp.	IHC	63
	ELISA	42, 67
Vibrio vulnificus biotype-2	ELISA	183
Viruses		
Infectious haematopoietic necrosis virus (IHNV)	FAT	60, 61
	ELISA	78
Viral haemorrhagic septicaemia (VHS)	ELISA	78, 184
Epizootic haematopoietic necrosis virus (EHNV)	ELISA	79, 185
Parasites		
Myxosporeans	IHC	65, 66

Immunodiffusion test has been used for detection of *R. salmoninarum*[51,52] but is not popular today due to availability of more sensitive methods.

Immunofluorescent techniques are attractive because of the rapidity of diagnosis. For example, detection of *R. salmoninarum* in fresh kidney imprints within 30 min by indirect fluorescent antibody test (IFAT) has been reported[42,53]. Application of fluorescent antibody test (FAT) and IFAT has been reported for bacteria such as *A. hydrophila*[54], *A. salmonicida*[55], *R. salmoninarum*[56–58], *Y. ruckeri*[59] and for viruses such as infectious haematopoietic necrosis (IHN) virus[60,61].

Immunohistochemistry (IHC) is a convenient method for detecting pathogens in tissue sections and this technique has been used with bacteria such as *A. salmonicida*[55], *R. salmoninarum*[62], *Mycobacterium* spp.[63], *Vibrio* spp.[64], *Yersinia ruckeri*[81] and for parasites such as myxosporeans[65,66].

ELISA-based methods are highly sensitive and permit quantitative assay of a large number of samples. Sandwich ELISA has been developed for detection of *A. salmonicida* in fish tissue[40]. For detection of carrier fish, ELISA was found to be more sensitive compared to culture and enhancement assays[40]. For pathogens such as mycobacteria which are difficult to culture, ELISA is a very attractive diagnostic tool. Adams *et al.*[42,67] developed sandwich ELISA using polyclonal antibodies to capture *Mycobacterium* spp. and a cocktail of three MAbs was used for detection. ELISA has been found to be a very sensitive diagnostic tool for detection of *A. salmonicida*[39,40,68,69], *Edwardsiella* spp.[70,71], *R. salmoninarum*[72–75], *Y. ruckeri*[69,76], *Vibrio* spp.[77] and for viruses such as IHN[78] and viral haemorrhagic septicaemia[78,79].

Western blot (WB) reaction permits analysis of several antigens in one assay and therefore would be helpful in resolving problems of cross reaction which is common with ELISA. This method has been applied for bacterial kidney disease caused by *R. salmoninarum*[80–82]. Griffiths *et al.*[81] noted that WB method provided sensitivity of pathogen detection comparable to that of drop-plate culture techniques in field trials.

Several serodiagnostic methods have been developed for diagnosis of shrimp diseases. MAb-based ELISA has been developed for pathogenic *Vibrio* spp. such as *V. vulnificus*[83], *V. alginolyticus*, *V. parahaemolyticus* and *V. harveyi*[84]. PAb-based diagnostic methods have been reported for few shrimp viruses. For detection of baculoviral midgut necrosis, Sano *et al.*[85] developed a fluorescent PAb test. ELISA-based PAb test for *Baculovirus penaei* has been reported by Lewis[86] while Tapay *et al.*[87] documented application of PAbs for the detection of rhabdovirus of penaeid shrimp. Though considerable effort has gone into development of MAbs to shrimp viruses, there has been limited success. Poulos *et al.*[88] noted problems with specificity of their MAbs with

IHHNV. In Western blots, their MAbs reacted specifically with purified IHHNV or its capsid proteins. However, in ELISA-based assays, their MAbs reacted non-specifically with normal shrimp tissue, leading to false positive results[88].

Disease management in aquaculture

Disease in the aquatic environment is the end result of a complex interaction among the host, the pathogen and the environment. Therefore strategies for disease management should consider all these three factors. Though disease prevention is the ultimate goal, it may be often difficult to achieve and therefore, therapeutic measures play a very important role in disease management. Antibiotics, fungicides and parasiticides have been used extensively in aquaculture. It has been estimated that not less than 38 antimicrobials and 22 parasiticides are being used in Asian aquaculture[89–91]. Ampicillin, erythromycin, furazolidone, nitrofurazone, oxolonic acid, oxytetracycline and sulpha drugs are some of the commonly used antibacterials while trichlorofen is the commonly used fungicide[91]. Malathion, formalin, malachite green, iodophor and benzalkonium chloride are used as parasiticides or as water sanitizers.

However, chemotherapeutic agents are coming under severe criticism for the environmental problems. Emergence of antibiotic-resistant pathogens[89,92,93] is a great problem and threat. Data from several authors indicate that the frequency of resistance reflects the pattern of antibiotic usage[94–96]. Similarly, reduction in frequency of resistance following reduction in the use of a particular antibiotic has also been reported[95,97,98]. Presence of R plasmid encoded resistance in fish pathogens has also been reported by many investigators[98–103]. There are also concerns on the antibiotic residues in cultured animals and on the accumulation of antibiotics in pond/marine sediments[93].

Disease management strategies based on vaccines and immunostimulants are being increasingly explored. A number of investigators have explored the possibility of protecting fish against disease using vaccines. Early reports on fish vaccines relate to vibriosis[104] and furunculosis[105]. Since then a number of studies have been conducted on the factors influencing the immune responses in fish. An important aspect to be considered is the ontogeny of immune response. Most studies indicate that the size of the fish at the time of immunization is critical for the development of optimum response[106,107]. In general, the larger and thus more mature the fish at the time of immunization, the greater the level and duration of protective immunity (Figure 2). The route of exposure to the immunizing antigen has a direct impact on the levels and types of protective immunity that develops[108]. Currently four methods are commonly used to deliver antigens. Injection (intraperitoneal, intramuscular or subcutaneous) induces highest levels of protection[109] but is very labour-intensive and stressful. Recently, semi-automated devices which can immunize 4000 fish per hour have been developed[110] and these reduce the stress on fish and risk of exposure to the worker. Vaccination by immersion is perhaps the most widely used method. In this method, fish are dipped for 20 s in a well-aerated vaccine suspension. It is possible to vaccinate 100 kg of fish per litre of vaccine[110]. Thus a litre of vaccine can be used to vaccinate 10,000 × 10 g fish. Dip vaccination would be stressful, but the problem is overcome by bath vaccination where fish are vaccinated by being exposed to higher dilutions of vaccines (e.g. 1 : 100) for times ranging from 20 min to several hours. Vaccine can be added directly to hatchery troughs or transport bags.

Spray vaccination is another modification of direct immersion but in this method fish must be handled thus making it stressful. The level of protection, though variable has been reported to be comparable to immersion vaccination[111]. Immunization by oral route by incorporating the bacterin in the feed is a potentially useful method[112]. Many factors are known to influence the ability of a vaccine to protect fish. These include environmental factors such as temperature and pollutants, host factors such as age and general health, husbandry factors such as handling, stress, diet, antibiotics and vaccine-related factors such as dose, nature of antigen, route of administration and presence of adjuvants.

Table 4 gives a summary of the status on vaccine development for fish. Warm water aquaculture in Asia has problems with several bacterial diseases such as motile aeromonads septicaemia, vibriosis, columnaris and

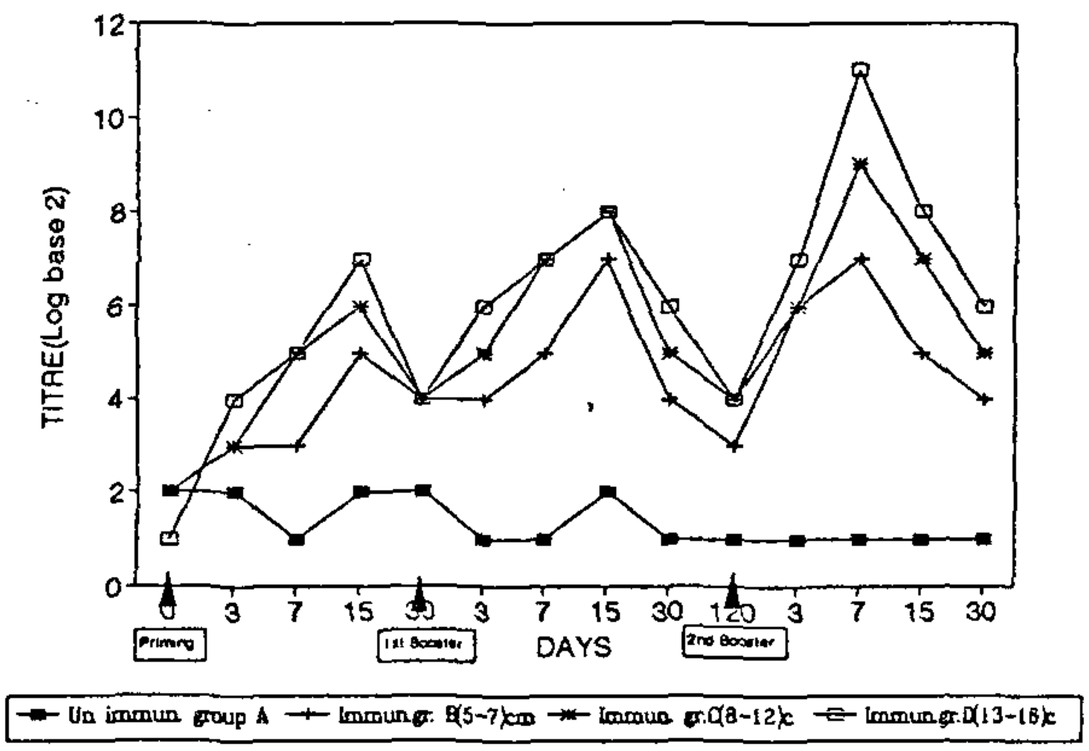

Figure 2. Influence of fish age on immune response. Indian major carp *Labeo rohita* of different sizes immunized with heat killed *Aeromonas hydrophila* antigen and immune response monitored by assessing agglutinating antibodies. Karunasagar *et al.*, unpublished results.

edwardsiellosis. Among these, diseases caused by motile aeromonads are most widespread. Motile aeromonads are implicated in a number of disease conditions such as septicaemias and ulcerative conditions[113–115]. Though commercial vaccine against motile aeromonads is not available, a number of experimental studies on immunization have been carried out. Following immunization using bacterin, significant protection against challenge has been observed in carp[116], Indian major carps[117,118], tilapia[119] and channel catfish[120]. The major problem with motile aeromonads is the heterogeneity in the strains, both biochemically and serologically. Karunasagar *et al.*[118] conducted studies on monovalent and polyvalent vaccines. The results suggest that monovalent vaccines made from some strains (G-49) conferred protection comparable to that obtained by polyvalent vaccines[118]. Interestingly with some strains, though the antibody levels were high, protection against challenge was not absolute, suggesting that factors other than agglutinating antibodies may be involved in protection against challenge. Alternatively antibodies against certain protective antigens may be important and these antigens of *A. hydrophila* have not yet been identified. Thus further studies are required before commercial vaccination against motile aeromonads becomes possible.

The possibility of using live attenuated vaccines has been investigated by a few investigators. Karunasagar *et al.*[117] noted that an aerolysin deletion mutant induced significant protection in challenge experiments. Moral *et al.*[121] developed an aromatic-dependent mutant of *A. hydrophila* as a candidate for live vaccine.

With vibriosis caused by *V. anguillarum*, the situation is less complicated because most disease outbreaks are due to serotype 01. This has made manufacture of the vaccine with wide applicability possible. Presently, a number of commercial vaccines are available[122]. Most studies indicate that formalin inactivated whole cell suspension applied by immersion provides consistent protection[123,124]. Vaccination by injection is also widely practised and for fish larger than 40 g, this has been reported to be the least expensive in spite of being labour intensive[109]. Some of the commercial vaccines

Table 4. Examples of fish vaccines and their status

Pathogens	Vaccine development status	Ref.
Bacteria		
Vibrio anguillarum	Several commercial vaccines available, most vaccines contain *V. anguillarum* serotype 1 and 2. Polyvalent vaccines with other antigens such as *V. salmonicida*, *A. salmonicida*, *V. ordalli* also available	122
Aeromonas salmonicida	Commercial vaccines available, effective with oil-based adjuvants, recombinant vaccines under development	129
A. hydrophila	No commercial vaccines available, several experimental studies, recombinant vaccines under development	118
Yersinia ruckeri	Commercial vaccine available	131
Edwardsiella ictaluri	Orally delivered killed vaccine licensed	128
Renibacterium salmoninarum	Effective vaccine yet to be reported	132
Pasteurella piscicida	No commercial vaccines, a polyvalent extracellular protein (ECP) enriched bacterin used in some countries	133
Viruses		
Infectious pancreatic necrosis virus	Inactivated viral vaccines give inconsistent results. Recombinant vaccines reported. Injectable vaccine based on viral protein VP2 licensed	137, 138
Viral hamorrhagic septicaemia	Injection of killed viral vaccine gives protection. Attenuated and recombinant vaccines under development	139
Infectious haematopoietic necrosis virus	Killed vaccines effective but too expensive. Recombinant vaccine based on glycoprotein has undergone field trials	140
Spring viraemia of carp	Commercial vaccine available. Killed and attenuated vaccines reported	141

are polyvalent and contain *V. anguillarum* 01, 02 or *V. ordalli*, *V. salmonicida*, *A. salmonicida* and *Y. ruckeri*[122].

Columnaris disease caused by *Flexibacter columnaris* affects both warm and cold water fish species[125]. Strains of *F. columnaris* are serologically homogeneous[126]. Experimental studies indicate that successful immunization against columnaris is possible[126] but as yet commercial vaccines are not available.

Edwardsiellosis due to *Edwardsiella tarda* has serious impact on cultured eel and seabream in Taiwan and *E. ictaluri* impacts channel catfish culture. Though *E. tarda* is serologically heterogeneous, generally only four serotypes are associated with disease in fish[127] and therefore, a polyvalent vaccine with a wide range of activity could be developed. Significant levels of protection could be achieved in eels by immersion vaccination. However, no commercial vaccines are yet available for *E. tarda*. Recently, an orally delivered commercial vaccine has been licenced[128].

Furunculosis is a major problem in temperate waters and several vaccines have been tried against the pathogen *A. salmonicida*. The data indicate that antibody responses to the polysaccharide capsule and iron-regulated outer membrane proteins are associated with protection[129]. Bacterins emulsified in oil adjuvants and delivered by intraperitoneal injection provide remarkably high degree of protection and this method is being adopted by a number of Atlantic salmon farmers. Techniques of genetic engineering have been used to develop live attenuated vaccines for furunculosis. Vaughan *et al.*[130] developed a mutant of *A. salmonicida* as a candidate for live attenuated vaccine.

Y. ruckeri bacterin was the first commercially produced fish vaccine and the formalin killed whole cell product continues to be highly effective whether administered by immersion, spray, injection or oral routes[131]. Though two serogroups are involved in disease epizootics, the vaccine contains only serovar 1 and the basis for cross protection afforded by the commercial vaccine to serogroup 2 is not understood[131].

Though bacterial kidney disease is a major problem in salmonid culture, an effective vaccine is yet to be developed against the causative agent, *R. salmoninarum*[132]. Being an intracellular organism, this presents a great challenge to vaccinologists. Serum antibodies alone are inadequate to deal with this pathogen and more needs to be learnt about cellular immunity and the means to induce such immunity. *Pasteurella piscicida* causing pasteurellosis in marine fish is a serologically homogenous organism. A number of vaccines have been tried and best protection has been reported with the extracellular product (ECP) enriched bacterin and this vaccine is being used in some European countries[133].

The impact of viral diseases in Asian aquaculture is largely unknown. In cold water fish culture, several viral diseases have been recorded. Apart from killed viral vaccines, biotechnological approaches such as pathogen attenuation, genetic recombination, protein engineering and subunit vaccines are popular in viral vaccine research, but presently these are very expensive and not yet applicable for mass production of biologicals for aquatic animals[134]. However, development of experimental viral vaccines using some of these procedures has been reported for infectious pancreatic necrosis virus (IPN), viral haemorrhagic septicaemia virus (VHS), infectious hematopoietic necrosis virus (IHNV), spring viraemia of carp and channel catfish virus[134–141]. Nevertheless only a killed virus vaccine for spring viraemia of carp is commercially available (Table 4).

Fish depend more heavily on non-specific defence mechanisms than mammals[142] and therefore immunostimulants have a significant role in health management strategies in aquaculture. There are at least 20 different compounds which have potential use as immunostimulants, adjuvants and vaccine carriers in fish[142]. Glucans from yeast have been observed to be good stimulators of non-specific defence mechanisms in fish[143]. In warm water fish, Indian major carp, *Labeo rohita*, yeast glucans have been observed to enhance phagocytic activity of leucocytes and stimulate generation of reactive oxygen species (ROS) in phagocytes. When glucans were administered along with *Aeromonas hydrophila* vaccine, the response was even more enhanced[144], suggesting that yeast glucans have important role in disease management in warm water aquaculture.

Immunoprophylaxis for shrimp is being seriously considered by various investigators but the knowledge about the immune system of shrimp is very limited. In addition to the hard exoskeleton which forms a structural and chemical barrier to pathogens and parasites, the crustaceans need an efficient internal immune defence network to deal with opportunistic or pathogenic micro-organisms which can gain entry into the body cavity through wounds or during molt. Like in other animals, crustacean host defence is largely based on the activities of the blood cells or haemocytes and haemolymph factors[145]. In most decapods, morphological criteria associated with biochemical assays have been used to identify and classify haemocytes. The three main types of circulating haemocytes have been identified and isolated by isopycnic cetrifugation on Percoll gradient[146]. Hyaline cells are characterized by the absence of granules, spreading ability and phagocytosis. The proportion of cell types varies with different species. In *Penaeus japonicus*, about 10% of the circulating haemocytes are classified as hyaline while no hyaline cells were found in *P. adspersus* and *Macrobrachium rosenbergii*[147]. Semigranular cells respond to microbial polysaccharides such as lipopolysaccharides and

β-1,3-glucans by degranulation[148]. Since the degranulated cells attach and spread on foreign surfaces, they have an important role in encapsulation[145]. Granular haemocytes with large granules are a repository for the prophenoloxidase (pro-PO) activating system. Exocytosis of this cell is triggered by two endogenous proteins associated with pro PO system, viz. 76 kD factor and the β-1,3-glucan binding protein which reacts with β-1,3-glucan[145].

Though phagocytosis is a very important cellular defence reaction, very little is known about the process in most crustaceans. While the involvement of haemolymph-borne opsonic factors has been suggested for freshwater crayfish, *Cherax destructor* and for the lobster, *Homarus americanus*, no evidence for such involvement was found in the shore crab *Carcinus maenas*[145]. However, enhanced phagocytic rate in the presence of haemocyte lysate suggested the involvement of pro PO system[149]. Nodule formation is an important mechanism for handling a large number of micro-organisms which become entrapped in several layers of haemocytes. The gills and hepatopancreas are thought to be the major sites of foreign body lodgement but little is known about the mechanisms by which foreign particles or microbes are dealt with after being engulfed or encapsulated by haemocytes[145]. Cytotoxicity for both tumour and nontumour cell lines has been demonstrated in haemocytes of freshwater crayfish, *Astacus astacus*[150]. The molecule responsible for this and the role of this activity in defence is not understood.

A number of haemolymph factors are also associated with defence reaction. These factors include naturally occurring or inducible bioactive molecules which agglutinate, precipitate or inactivate non self-particles and those that have bactericidal, lytic or bacteriostatic properties[151].

Agglutinins which cause aggregation or agglutination of foreign particles have been reported from a number of crustacean species[151]. Though agglutinins occur naturally, enhanced titres after exposure to test materials has been reported for *P. monodon*[152]. However, the effect was small, short-lived and non-specific. Agglutinins would help sequestration of invasive organisms from the haemolymph and therefore would contribute to disease resistance. Lectins, which are generally recognized by their ability to cause haemagglutinations, are also found in many crustacean species. The function of these haemagglutinins in defence is not understood. It is suspected that lectins serve as recognition molecules. Ratanapo and Chulavatnatol[153] have purified monodin, a lectin from *P. monodon*. Using specific antibody, Bachere *et al.*[154] have localized this molecule in semi-granular haemocytes of *P. japonicus*, suggesting the secretory nature of this lectin. An α-2-macroglobulin-like protein has been identified in shrimp using monoclonal antibody[154]. This protein appears to be a dimer of 340 kD and localized in the plasma and in the membrane of all the haemocyte types and in the vesicular inclusions of hyaline and semi-granular cells[155]. The function of this protein is largely unknown. In mammals, the α-2-macroglobulin binds defensins, small cationic peptides with antimicrobial and cytotoxic properties and the formed complex is internalized by receptors on macrophages[154]. These molecules may play a key role in the regulation of defence and inflammatory process.

Microbicidal factors have also been reported from crustaceans. Bactericidins have been described in crabs and lobsters[151]. In some cases, killing activity has been found to be induced by pretreatment of the host with live or heat-killed bacteria, but the responses have limited specificity and the time taken to reach maximum effect ranges from 36 h to 7 days[151]. Microbicidal factors may also be associated with different tissues and haemocytes. Biochemically little is known about these killing factors. Lysozyme and peroxidase-mediated antimicrobial activity seems to be absent in crustaceans. So far molecules similar to antimicrobial peptides described in lipido- pteran insects[156] have not been demonstrated, but it is possible that they exist in crustaceans[151]. Melanin and its intermediaries generated through the prophenoloxidase system have been shown to have antimicrobial activity by inhibiting extracellular proteinases and chitinases[145].

Clotting factors play an important role in defence. In crustaceans, clotting is mediated by coagulogens present in the plasma and also compartmentalized within circulating cells[151]. The plasma factor is converted to covalently linked polymers of coagulin by Ca^{2+}-dependent transaminase whereas the cell factor is converted to a gel by a serine protease proclotting enzyme which may be triggered by microbial molecules such as lipopolysaccharide (LPS) and β-1,3-glucans[151]. A plasma clotting protein from the shrimp, *P. japonicus* has been identified using monoclonal antibody and characterized as a 360 kD protein composed of two disulphide-bonded subunits of 180 kD (ref. 154). Activation of clotting cascade in response to LPS or β-1,3-glucans results in the formation of a clot which entraps the invading pathogens. This process is also linked to the triggering of prophenoloxidase activating system[145].

As discussed above, the crustacean immune system is heavily dependent on nonspecific factors and the specific immune system that is seen in vertebrates seems to be absent. Nevertheless, several investigators have studied the possibility of enhancing the immune response and disease resistance by exposure to microbial products (Table 5). Induction of bactericidins, agglutinins and protection against challenge by pathogens has been reported for freshwater crayfish[157], spiny lobster[158], *Squilla mantis*[159] and *P. monodon*[152,160–163]. Adams[152] noted induction of bactericidins and other humoral factors within day 1 of injection of heat-killed *V. alginolyticus*. Bactericidins peaked at day 2 and persisted until day 5.

Table 5. Examples of induction of immune response in crustaceans

Crustacean species	*Inducing substance*	Response induced	Ref.
Crayfish (*Parachaerops bicarinatus*)	Formalized cells of *Pseudomonas*	Protection against injection	157
Spiny lobster (*Panulirus argus*)	Bacterin	Bactericidin	158
Squilla mantis	Vibrio bacterin	Bactericidal and agglutination activity	159
Penaeus monodon	V. alginolyticus bacterin	Bactericidal activity	152
P. monodon	Glucan	Protection against *V. vulnificus* challenge	160
P. monodon	Vibrio bacterin, yeast β-1,3 glucan	Vibriocidal activity, polyphenol oxidase activity. Reactive oxygen species induction	162, 163, 165–167
P. monodon	Vibrio bacterin, yeast β-1,3 glucan	Improved resistance to challenge by *Vibrio* spp., white spot baculovirus	162, 163, 165–167
P. monodon	Peptidoglycan	Increased phagocytic index, improved protection against WSSV	168

Immunostimulation of *P. monodon* by glucans has also been reported[160,164]. In our laboratory we have studied the effect of heat-killed *V. harveyi* and yeast β-1,3-glucan. Our results indicate that treatment with either of these alone induces immune response assessed by vibriocidal activity in haemolymph and haemocyte, polyphenoloxidase activity in haemolymph and haemocytes and generation of reactive oxygen species in haemocytes[161]. The response in all the parameters was highest in shrimp treated with the mix of vibrio bacterin and yeast β-1,3-glucans. However, the effect was short lived (5–7 days) and lacked 'secondary response'-like effect after second contact. Nevertheless the immune system could be stimulated by weekly administration of immunostimulant. Trials of this immunostimulant were carried out in shrimp hatcheries and farms. In hatcheries, immunostimulant treatment was observed to enhance survival from nauplius to post larva 20 (PL 20) by 20% (ref. 165). In growout system, regular treatment with immunostimulant (once in five days through feed) resulted in survival of over 85% in a 15 ha farm infected with white spot baculovirus[166]. These results suggest that though the shrimp immune system is non-specific, it would be possible to enhance disease resistance in shrimp by careful regular use of immunostimulants. Studies by Song *et al.*[167] and Itami *et al.*[168] also show that disease resistance in penaeid shrimps can be enhanced by treatment with microbial cell components. Thus there is a potential to use immunostimulants in aquaculture but a lot of studies are necessary to understand the mechanism of their action.

It is also to be noted that the crustacean immune system is highly influenced by environmental factors[151] and these factors should be considered while planning disease management strategies. Nevertheless, during the coming years, vaccines and immunostimulants are sure to have great potential to make aquaculture sustainable.

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