Disease Control in Fish and Shrimp Aquaculture in Southeast Asia - Diagnosis and Husbandry Techniques

Yasuo Inui and Erlinda R. Cruz-Lacierda
Editors

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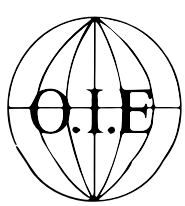
Disease Control in Fish and Shrimp Aquaculture in Southeast Asia - Diagnosis and Husbandry Techniques

Proceedings of the SEAFDEC-OIE Seminar-Workshop on Disease Control in Fish and Shrimp Aquaculture in Southeast Asia-Diagnosis and Husbandry Techniques

4-6 December 2001, Iloilo City, Philippines

Yasuo Inui and Erlinda R. Cruz-Lacierda
Editors



Disease Control in Fish and Shrimp Aquaculture in Southeast Asia - Diagnosis and Husbandry Techniques

On the Cover

1 2

1. Grouper with VNN (Photo by LD de la Peña)
2. Shrimp with WSSV (Photo by LD de la Peña)

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FOREWORD

Aquaculture is recognized as the fastest growing food producing sector in the world. With the global population still increasing and capture fisheries leveling off, aquaculture is seen in the future as the hope to fill the gap between fish supply and demand. Aquaculture production in Southeast Asia has contributed significantly to worldwide supply and, in the process, to the countries' economic development. However, over the last ten years the rapid and generally unregulated development of aquaculture led to frequent occurrence of infections diseases, threatening the sustainability of the industry in the region. It is in this context that SEAFDEC Aquaculture Department implemented the "Fish Disease Project" funded by the Government of Japan Trust Fund since the year 2000.

This "Seminar/Workshop on Disease Control in Fish and Shrimp Aquaculture in Southeast Asia-Diagnosis and Husbandry Techniques" held in Iloilo City on 4-6 December 2001, as one of the components of the Project, was co-organized by SEAFDEC and OIE. The objectives of the workshop were: (1) to review the current research studies and diagnostic techniques on viral diseases of shrimp and marine fish in Southeast Asia; (2) to identify an appropriate training program for the Fish Disease Project; and (3) to review the current research on techniques in controlling shrimp vibriosis.

It was a great honor for SEAFDEC AQD to host the Seminar/Workshop especially with the presence of leading fish disease experts not only from Southeast Asia but from other regions of the world as well. Special acknowledgement is given to OIE for co-organizing this activity, to other collaborating institutions for their active participation, and to the Government of Japan for the financial support.

We are therefore pleased to make available this Proceedings. It is our hope that this will contribute to a sustainable aquaculture development in Southeast Asia.

Rolando R. Platon
Chief
SEAFDEC/AQD

PREFACE

Aquaculture production in Southeast Asia has grown rapidly in the past 10 years and has been contributing significantly to the worldwide food supply. However, the rapid and generally uncontrolled development of aquaculture led to frequent occurence of infectious diseases, which have been threatening the sustainability of aquaculture in the region. The newly emerging viral diseases, represented by monodon baculovirus (MBV) and white spot syndrome virus (WSSV) have become a serious impediment to shrimp aquaculture. So are bacterial diseases, primarily luminous vibriosis in shrimp. There is also an increasing danger due to viral nervous necrosis (VNN) and iridovirus disease in marine fish aquaculture.

The Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC AQD) has been implementing the "Regional Fish Disease Project" under the auspices of the Japanese Trust Fund since year 2000. This Seminar/Workshop is one of the components of the Project and has been organized by SEAFDEC AQD and the Office International des Epizooties (OIE). The Seminar/Workshop has:

1. reviewed the current research studies and diagnostic techniques on viral diseases of shrimp and marine fish in Southeast Asia;
2. reviewed the current research studies on techniques in controlling shrimp vibriosis; and
3. identified an appropriate training program for the Project.

The resource persons of this Seminar have made thorough review of their assigned subjects; thus, making the contributed papers an excellent introduction to the current researches and disease control measures. We hope this volume will serve as a guide in planning the direction of the disease control program in the region. We look forward to these challenges.

We thank Mr. Shogo Sugiura, Deputy Secretary-General of SEAFDEC and Mr. Teruhide Fujita, Regional Representative of OIE Regional Representation for Asia and the Pacific, for bringing their institutions together for this Seminar-Workshop.

Yasuo Inui

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DIAGNOSTIC TECHNIQUES FOR VIRAL DISEASES OF MARINE FISH AND SHRIMP

Advances in Diagnosis and Management of Shrimp Virus Diseases in the Americas

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ABSTRACT

The most important diseases of cultured penaeid shrimp, in terms of economic impact, in Asia, the Indo-Pacific, and the Americas, have infectious etiologies. Although diseases with bacterial, fungal, and parasitic etiologies are also important, certain virus-caused diseases stand out as the most significant. The pandemics due to the penaeid viruses WSSV, TSV, YHV, and IHHNV have collectively cost the penaeid shrimp industry billions of dollars in lost crops, jobs, and export revenue. Although not as sudden nor as catastrophic in their onset and course, certain bacterial, fungal, and parasitic diseases of shrimp have also been responsible for very significant production losses, and the relative importance of many of these diseases should not be discounted.

The social and economic impacts of the pandemics caused by WSSV and TSV have been especially profound in the Americas, and in the wake of these viral pandemics the shrimp culture industry has sought ways to restore the industry's levels of production to the "pre-virus" years. Central to improving disease) prevention and management strategies is the incorporation of the concepts of biosecurity into shrimp farm design and operational strategies. Disease management in shrimp aquaculture is an important component to biosecurity of farms and to the sustainability of individual farms, shrimp farming countries, or entire geographic regions. The first step in disease management requires the availability of accurate and reliable diagnostic methods and knowledge of the biology of the diseases of concern. The recognition of the need for biosecurity and disease management in the Americas is reflected in the recent proliferation of shrimp disease diagnostic laboratories in the Americas. Where there were only a handful of shrimp disease diagnostic laboratories a decade ago, there are 40 or more such laboratories serving the industry today.

Diagnostic methods may be applied to determining the cause of disease(s) that are adversely affecting the culture performance or survival of farmed shrimp stocks or they may be used for surveillance purposes to screen for the presence of specific pathogens in otherwise healthy shrimp for the purpose of disease control. As diagnostic methods have improved and become more widely available, the interest in culturing specific pathogen-free (SPF) shrimp stocks in biosecure facilities has increased markedly in many regions in the Americas. The methods being used in shrimp disease diagnostic laboratories in the Americas were recently surveyed. Of the 40 laboratories contacted, 27 responded to the survey. Approximately 75% of the labs responding to the survey provide diagnostic services using both molecular (PCR, RT-PCR and gene probes) and classical (routine histology and microbiology) methods, while nearly all (93%) of the diagnostic labs offer diagnostic testing and screening services based on molecular methods (i.e. assays with gene probes and PCR/RT-PCR).

INTRODUCTION

The global penaeid shrimp farming industry is nearly 30 years old and it produced about 865,000 metric tons of whole shrimp in 2000 from its farms (Rosenberry, 2001). The importance of the industry to the global economy is reflected in these production numbers and by the millions of persons employed directly or indirectly by the industry. That farmed shrimp are among the most important foreign exchange earners for many tropical and subtropical coastal nations further documents the economic and social importance of the industry. Certain diseases have had a profound effect on penaeid shrimp aquaculture. Rosenberry (2001) estimated that disease due to the white spot syndrome virus (WSSV) "robbed the industry" of approximately 200,000 metric tons of production in 2000 worth more than $1 billion. WSSV and other infectious agents have been and continue to be significant impediments to the development and sustainability of the industry.

Disease in shrimp farming may be defined as any adverse condition due to biotic (living or infectious) agents or abiotic (non-living) agents that adversely affects culture performance (Lightner, 1996a). Biotic diseases of shrimp are those that have living agents as the cause, while abiotic diseases may be caused by environmental or physical extremes (temperature, hypoxic conditions, nitrogen supersaturation, extremes of pH, etc.), chemical toxicants, pesticides, nutritional deficiencies or imbalances, improper handling, etc. Most biotic diseases have infectious etiologies, and the list of biotic diseases affecting shrimp is not too different from the list of diseases that affect other animals. Many of the major causes of any kind of disease in vertebrate animals are represented in penaeid shrimp. Shrimp have infectious diseases caused by viruses, rickettsia, true bacteria, protozoan and helminth parasites, etc. They have benign and neoplastic tumors and they develop nutritional diseases when fed inadequate diets (Lightner, 1988, 1993a, 1993b, 1996a).

The terms "disease agent" (= etiological agent or pathogen) and "disease" may be defined differently by biomedical and aquaculture pathology professionals. There is an ongoing debate as to whether non-clinical infections (*i.e.* the presence and reproduction of a pathogen in a host) constitute a positive diagnosis of disease. The terms as used in this review are according to the definitions given in the Aquatic Animal Health Code published by the International Office of Epizootics (OIE, 2000a), the administrative arm of the World Animal Health Organization. For the sake of clarity, these OIE definitions are given here:

Disease - means clinical or non-clinical infections with one or more etiological agents of the diseases listed in the OIE Code.

Disease agent - means an organism that causes or contributes to the development of a disease listed in the OIE Code.

With these definitions it is apparent that OIE defines *disease* to include non-clinical infections by particular pathogens, as well as clinical infections which may be accompanied by high mortality rates. Therefore, the detection of particular pathogen in a diagnostic assay is a positive case of the *disease* caused by that disease agent.

The most important diseases of cultured penaeid shrimp, in terms of economic impact, in Asia, the Indo-Pacific, and the Americas have infectious agents as their cause (Tables 1 and 2).

Table 1. Major diseases of Indo Pacific and east Asian penaeid shrimp

Viral Diseases	Bacterial and Fungal Diseases	Other Diseases
White Spot Syndrome Virus Yellow Head Virus group BMN group MBV group IHHNV HPV group REO group	Vibriosis: - septic HP necrosis - hatchery vibriosis - luminescent vibrio Other bacteria: - Rickettsia Fungal: - Larval mycosis - Fusariosis	Epicommensals and parasites: - *Leucothrix mucor* - peritrich protozoans - gregarines - microsporidians Nutritional imbalances Toxic syndromes and environmental extremes

Table 2. Major diseases of the American penaeid shrimp

Viral Diseases	Bacterial and Fungal Diseases	Other Diseases
White Spot Syndrome Virus Taura Syndrome Virus IHHNV BP group HPV group REO III? LOW? RPS? Yellow Head Virus?	Vibriosis: - 'Sindrome Gaviota' - hatchery vibriosis - luminescent vibrio - shell disease Other bacteria: - NHP bacterium Fungal: - Larval Mycosis - Fusariosis	Epicommensals and parasites: - *Leucothrix mucor* - peritrich protozoans - gregarines - microsporidians Nutritional imbalances Toxic syndromes and environmental extremes Zoea II syndrome

Among the infectious diseases of cultured shrimp, certain virus-caused diseases stand out as the most significant. Some of the most important diseases (and their etiological agents) were once limited in distribution to either the Western or Eastern Hemisphere (Fulks and Main, 1992; Lightner, 1996a). However, the international movement of live (for aquaculture) and dead (commodity shrimp for reprocessing and commerce) has led to the transfer and establishment of certain pathogens from one hemisphere to the other (Lightner 1996b; Lightner *et al*., 1995; Durand *et al*., 2000; AQUIS, 2000). WSSV was moved from Asia to the Americas by this route and TSV was moved in the opposite direction (Nunan *et al*., 1998a; Tu *et al*., 1999; Durand *et al*., 2000). Perhaps these transfers and introductions could have been prevented if the industries and governments of the exporting and importing countries had known of the risks posed by their actions and if the appropriate disease diagnostic and pathogen detection methods had been readily available when the most damaging transfers were being made. Many of the most significant shrimp pathogens were moved from the regions where they initially appeared to new regions even before the "new" pathogen had been recognized, named, proven to cause the disease, and before reliable diagnostic methods were developed. The pandemics due to the penaeid viruses WSSV and TSV, and to a lesser extent to IHHNV and YHV, cost the penaeid shrimp industry billions of dollars in lost crops, jobs, and export revenue well before their etiology was understood (Table 3). The social and

economic impacts of the pandemics caused by these pathogens in countries in which shrimp fanning constitutes a significant industry have been profound. In the wake of the viral pandemics the shrimp culture industry has sought ways to restore the industry's levels of production to the "pre-virus" years. The application of biosecurity to shrimp farming, coupled with improved disease diagnostic methods and support, is central to those efforts.

Table 3. Estimated economic losses since the emergence or introduction of diseases due to WSSV, TSV and IHHNV in the penaeid shrimp aquaculture industry of the America:

Virus	Year of emergence to 2001	Product loss (dollars)
WSSV-Americas	1999	1-2 billion
TSV	1991-92	1-2 billion
IHHNV*	1981	0.5-1.0 billion

* Includes Gulf of California fishery losses for 1989-1994.

This paper reviews the current status of diagnostic methods and infrastructure in the Americas and its application with certain of the concepts and principles of biosecurity to shrimp disease management strategies in the Americas. Shrimp taxonomy used in this review is according to Holthuis (1950).

CURRENT DIAGNOSTIC METHODS

Modem penaeid shrimp diagnostic and research laboratories are based on traditional methods of disease diagnosis and pathogen detection that have been adapted from methods used in fish, veterinary and human diagnostic laboratories. Methods for the detection of pathogens and the diagnosis of diseases that are currently in use by shrimp pathologists and by diagnostic labs have been reviewed many times in the past decade (Baticados, 1988; Baticados *et al*., 1990; Brock, 1991, 1992; Brock and Lightner, 1990; Brock and LeaMaster, 1992; Brock and Main, 1994; Flegel *et al.* 1992; Fulks and Main, 1992; Johnson, 1990, 1995; Lightner, 1988, 1993a, 1993b, 1996a, 1999a; Lightner and Redman, 1991, 1992, 1998; Lightner *et al*., 1992a, 1992b, 1994; Limsuwan, 1993;Liu, 1989; OIE, 2000a, 2000b). In penaeid shrimp pathology, diagnosticians rely heavily on case history, gross signs and behavior, morphological pathology (direct bright-field or phase contrast light microscopy and electron microscopy) and classical microbiology (bacteriology and mycology) (Table 4). Among the most important of these are gross and clinical signs, with the most commonly applied laboratory tests being direct examination and microscopy using the light microscope, classical microbiology with isolation and culture of the agent, and routine histology and histochemistry (Bell and Lightner, 1988; Lightner, 1996a). Virtually every functional shrimp pathology/diagnostic laboratory today is equipped to do direct light microscopic methods and routine procedures in histology and bacteriology. Paradoxically, important techniques involving tissue and cell culture, hematology and clinical chemistry, which are virtual cornerstones of vertebrate biomedical research, diagnostics, and pathology, have either not been successfully applied as routine diagnostic tools in penaeid shrimp pathology (in the case of tissue culture), or have not

Table 4. Methods available to diagnosticians for shrimp disease diagnosis and pathogen detection

Method	Tests and Data Obtained
History	History of disease at facility or in region, facility design, source of seed stock (*e.g.* wild or domestic specific pathogen-free, SPF, or resistant, SPR), type of feed used, environmental conditions, etc.
Gross, clinical signs	Lesions visible, behavior, abnormal growth, feeding or food conversion efficiency, etc.
Direct microscopy	Bright-field, phase contrast or dark-field microscopic examination of stained or unstained tissue smears, whole-mounts, wet-mounts, etc., of diseased or abnormal specimens
Histopathology	Routine histological or histochemical (with special stains) analysis of tissue sections
Electron microscopy	Ultrastructural examination of tissue sections, negatively stained virus preparations, or sample surfaces
Culture & biochemical identification	Routine culture and isolation of bacterial isolates on artificial media and identification using biochemical reactions on unique substrates.
Enhancement	Rearing samples of the appropriate life stages of shrimp under controlled, stressful conditions to "enhance" expression of latent or low grade infections
Bioassay	Exposure of susceptible, indicator shrimp to presumed carriers of a pathogenic agent
Antibody-based methods	Use of specific antibodies as diagnostic reagents in immunoblot, immunohistochemistry, agglutination, IFAT, ELISA, or other tests
Hematology & clinical chemistry	Determination of hemocyte differential count, hemolymph clotting time, glucose, lactic acid, fatty acids, certain enzymes, etc.
Toxicology/Analysis	Detection of toxicants by analysis and verification of toxicity by bioassay
DNA probes	Detection of unique portions of a pathogen's nucleic acid using a labeled DNA probe
PCR/RT-PCR	Amplification of unique sections of a pathogen's genome to readily detectable concentrations using specific primer pairs
Tissue culture	*In vitro* culture of shrimp pathogens in non-shrimp tissue culture systems or in primary cell cultures derived from shrimp

provided routinely practical diagnostic data (in the case of hematology and clinical chemistry) (Crane and Benzie, 1999). Likewise, the development of antibody-based diagnostic methods for penaeid shrimp diseases has not been remarkable until recently (Lightner 1999a), when methods based on pathogen detection using monoclonal antibodies were developed (Poulos *et al.* 1999, 2001). Even more significant have been the development of molecular methods (using gene probes and PCR/RT-PCR), which have been found to provide accurate and standardizable methods for disease diagnosis and pathogen detection to the penaeid shrimp culture industries, especially for certain penaeid viruses (Chang *et al*., 1993; Lo *et al*. 1996, 1997; Lightner, 1996a, 1999a, 1999b; Mari *et al*., 1998; OIE 2000b; Tang and Lightner, 1999) (Tables 5-7).

Table 5. Diagnostic and pathogen detection methods for the OIE notifiable and listed viral diseases of penaeid shrimp (modified from Lightner, 1996a; 1999a; Lightner and Redman, 1998)

Method*	**WSSV**	**IHHNV**	**BP**	**MBV**	**BMN**	**SMV**	**YHV-group**	**TSV**
Direct BF / LM / PH / DF	++	-	+++	+++	++	-	++	+
Histopathology	++	++	++	++	++	++	+++	+++
Bioassay	++	+	+	-	+	-	+	++
TEM / SEM	+	+	+	+	+	++	+	+
ELISA / IHC with PAbs or MAbs	+++	-	+	-	+	-	+/-	++
DNA Probes DBH / ISH	+++	+++	++	++	++	+++	+++	+++
PCR / RT-PCR	+++	+++	+++	+	-	+++	+++	+++

* Definitions for each virus:

– = no known or published application of technique

\+ = application of technique known or published, but not commonly practiced or readily available

++ = application of technique considered by authors of present paper to provide sufficient diagnostic accuracy or pathogen detection sensitivity for most applications

+++ = technique provides a high degree of sensitivity in pathogen detection

Methods: BF = bright field LM of tissue impression smears, wet-mounts, stained whole mounts; LM = light microscopy; PH = phase microscopy; DF = dark-field microscopy; EM = electron microscopy of sections or of purified or semi-purified virus; ELISA = enzyme = linked immunosorbent assay; IHC = immunohistochemistry; PAbs = polyclonal antibodies; MAbs = monoclonal antibodies; DBH = dot blot hybridization; ISH = *in situ* hybridization; PCR = polymerase chain reaction; RT-PCR = reverse transcriptase PCR

Molecular diagnostic methods have become as important as classical methods (such as routine histopathology and microbiology) to the shrimp culture industry in recent years (Lightner, 1999a; OIE, 2000a, 2000b; Vanpatten and Lightner 2001). Methods employing gene probes PCR/RT-PCR have recently developed and applied to the diagnosis of certain infectious diseases of penaeid shrimp. Development and application of the first gene probe to the diagnosis of the shrimp virus IHHNV was reported only 8 years ago (Lightner *et al*., 1999b; Mari *et al*., 1993a). When labeled with (what was once traditional) radioactive tags, the use of gene probes was an option for only the best equipped diagnostic and research laboratories. However, the application of non-radioactive labeling methods has made gene probe technology readily available to shrimp research and diagnostic laboratories. The first non-radioactive gene probes for shrimp pathology were developed employing the non-radioactive Genius TMI Kit (Boehringer Mannheim, not dated), which contains digoxigenin-11-dUTP (DIG) as the DNA label and uses an ELISA-based system for final detection (Lightner *et al*., 1994; Mari *et al*., 1993a). This led to the development of the non-radioactive DIG-labeled gene probes for IHHNV and to their commercial application in diagnostic kits marketed under the product name 'ShrimProbes™' by DiagXotics (Wilton, CT, U.S.A.). Now the industry has available from commercial sources molecular tests using non-radioactively labeled DNA probes and PCR/RT-PCR methods for many of the more significant diseases of penaeids (Table 7).

Table 6. Summary of methods for surveillance and confirmatory diagnosis of OIE Notifiable and Listed penaeid shrimp viral pathogens

AGENT	SURVEILLANCE	CONFIRMATORY DIAGNOSIS
TSV	RT-PCR	RT-PCR, DNA probes, AB, histology
WSSV	PCR, AB	PCR, DNA probes, AB, histology, bioassay
YHV	RT-PCR	RT-PCR, DNA probes, AB, histology, bioassay
BMN	histology	Direct microscopy, histology
BP/MBV	PCR, direct microscopy, histology	Direct microscopy, histology, PCR
IHHNV	PCR, DNA probes	PCR, DNA probes, histology
SMV	none	DNA probes, histology, bioassay

Table 7. Commercially available molecular and antibody-based diagnostic products for penaeid shrimp pathogens and their applications*

Pathogen	DNA-based Tests			Antibody-based Tests	
	Dot Blot	In *situ*	PCR	Immuno Dot	Immuno-histochemistry
WSSV	X	X	X	X	X
IHHNV	X	X	X		
TSV	X	X	X	X	X
HPV	X	X	X		
MBV	X	X	X		
BP	X	X	X		
YHV	X	X	X		
NHP bacterium	X	X	X		

* Available from DiagXotics, Inc. Wilton, CT, USA.

Since the first gene probe to the shrimp parvovirus IHHNV was developed in 1992 (Mari *et al*., 1993a), the technology has been applied to the development of additional gene probes to other shrimp viruses, a rickettsia-like bacterium and a microsporidian (Table 7). At the present time, DIG-labeled gene probes have been developed and are available for IHHNV, HPV, TSV, BP, MBV, WSSV, and YHV (Tables 5 and 8) (Bruce *et al*., 1993; Durand *et al*., 1996; Flegel *et al*., 1996; Lightner, 1996a; Lightner *et al*., 1994; Mari *et al*., 1993b, 1995; Nunan and Lightner, 1997;Poulos *et al*., 1994; Wang *et al*., 1995). Using essentially the same technology, additional DIG-labeled gene probes have been developed for the causative agent of necrotizing hepatopancreatitis, an intracellular, rickettsial-like bacterium (Frelier *et al*., 1992, 1993, 1994; Krol *et al*., 1991; Lightner *et al*., 1992c; Lightner, 1996a; Loy and Frelier, 1996; Loy *et al*., 1996), and for the microsporidian *Agmasoma* sp., which parasitizes *P. monodon* and *P. merguiensis* in southeast Asia (Pasharawipas and Flegel, 1994; Pasharawipas *et al*., 1994). Many of these probes are commercially available as DIG-labeled probes or in kit form (Table 7).

The polymerase chain reaction (PCR) has had numerous recent applications to pathogen detection and shrimp pathology research (Tables 5-7). In PCR, small, otherwise undetectable, amounts of DNA can be amplified to produce detectable quantities of the target DNA. This is accomplished by using specific oligonucleotide primers designed for the target DNA sequence. The resultant PCR product may then be compared to a known standard using gel electrophoresis, by reaction with a specific DNA probe of PCR products blotted directly onto a membrane or to the PCR products in Southern transfers. In some applications PCR products themselves may be labeled with DIG and used as specific DNA probes (Innis *et al*., 1990; Perkin Elmer, 1992). When DNA sequence information is known for specific nucleic acid sequences (of penaeid shrimp viruses, bacteria, etc.) primers can be synthesized to target specific nucleotide sequences. The unique target sequences may belong to a virus, a bacterium, or to any nucleic acid sequence. Various computer programs exist which aid in selection of optimal primers, provided target DNA sequence information is available (Innis *et al*., 1990; Perkin Elmer, 1992).

PCR has been applied to research and pathogen detection for most of the shrimp viruses of concern to modem day shrimp culture (Tables 5-7) (Lightner, 1999b; Nunan *et al*., 1998b, 2000; OIE, 2000b; Wang *et al*., 1996; Walker and Subasinghe, 1999). Other applications of PCR to shrimp pathology research and pathogen detection include reports of the application of PCR to the detection of bacterial pathogens such as the NHP bacterium (Loy *et al*., 1996b) and *Vibrio penaeicida* (Genmoto *et al*., 1996).

There is a growing need to standardize and validate the DNA-based diagnostic methods and the laboratories that use them (Walker and Subasinghe, 1999). Standardization of DNA-based diagnostic methods is almost inherent in the nature of the tests. That is, a specific DNA probe, or a specific set of primers, that is used to demonstrate the presence or absence of a unique DNA or RNA sequence does not vary from batch to batch. Hence, with proper controls, these DNA-based methods are readily standardized (Reddington and Lightner, 1994). However, despite the growing dependence of the shrimp culture industry on DNA-based diagnostic methods, none of the tests that are available from commercial sources nor from the literature have been validated using controlled field tests. Likewise, there are no formal accreditation or certification programs yet in place to assure that test results from technicians and laboratories running the tests are indeed accurate and properly controlled (Lightner and Redman, 1998; Lotz and Lightner, 1999; Lightner, 1999b). The implementation of a formal program by appropriate international agencies or professional societies is needed to validate

Table 8. Viruses of Penaeid Shrimp (as of July 2001; modified from Lightner, 1996; 1999a; Lightner and Redman, 1998)

Family Group/Acronym/ Full Name		
PARVOVIRUSES (Parvoviridae):		
IHHNV	=	infectious hypodermal and hematopoietic necrosis virus
HPV	=	hepatopancreatic parvovirus
SMV	=	spawner-isolated mortality virus
LPV	=	lymphoidal parvo-like virus
BACULOVIRUSES and BACULO-LIKE VIRUSES:		
BP-type	=	*Baculovirus penaei* type viruses (PvSNPV type sp.):
		BP strains from the Gulf of Mexico, Hawaii & Eastern Pacific
MBV-type	=	*Penaeus monodon*-type baculoviruses (PmSNPV type sp.):
	=	MBV strains from East & SE Asia, Australia, & Indo-Pacific
BMN	=	from *P. japonicus* in Japan
PHRV	=	hemocyte-infecting non-occluded baculo-like virus
WHITE SPOT SYNDROME VIRUS (Nimaviridae, p.n.f.) (WSSV and synonyms):		
SEMBV	=	systemic ectodermal & mesodermal baculo-like virus
RV-PJ	=	rod shaped virus of *P. japonicus*
PAV	=	penaeid acute viremia virus
HHNB	=	hypodermal & hematopoietic necrosis baculo-like virus; agent of
	=	"SEEDS" (shrimp explosive epidermic disease)
WSBV	=	white spot baculo-like virus
PRDV	=	penaeid rod-shaped DNA virus
WSSV	=	white spot syndrome virus
WSV	=	white spot virus
IRIDOVIRUS:		
IRIDO	=	shrimp iridovirus
RNA VIRUSES		
PICORNAVIRUS (Picornaviridae):		
TSV	=	Taura syndrome virus
REOVIRUSES:		
REO-III & IV	=	reo-like virus type II and IV
TOGA-LIKE VIRUS:		
LOVV	=	lymphoid organ vacuolization virus
RHABDOVIRUS:		
RPS	=	rhabdovirus of penaeid shrimp
YELLOW HEAD VIRUS GROUP (*Roniviridae*, p.n.f.):		
YHV/"YBV"	=	yellow head virus of *P. monodon*
GAV	=	gill associated virus of *P. monodon*
LOV	=	lymphoid organ virus of *P. monodon.*

new diagnostic methods and to periodically review the accreditation and certification of diagnosticians and diagnostic laboratories. The establishment of regional reference laboratories for DNA-based diagnostic methods of penaeid shrimp/prawn pathogens would fit well into such a program with the goal of making these methods uniform, reliable, and readily applicable to disease control and management strategies for viral diseases of cultured penaeids.

SURVEY OF SHRIMP DISEASE DIAGNOSTIC LABS IN THE AMERICAS

The application of effective pathogen detection and disease diagnostic methods by industry are essential to better understand and prevent losses due to disease. Where there were only a handful of shrimp disease diagnostic laboratories a decade ago in the Americas, there are 40 or more such laboratories serving the industry today. A survey was carried out at the request of the Global Aquaculture Alliance to provide a snapshot of the current capabilities of a representative sample of laboratories in the Americas in terms of diagnostic infrastructure and the types of services provided (Vanpatten and Lightner, 2001). The findings of that survey are summarized here.

Laboratory characteristics

Of the approximately 40 diagnostic labs in the Americas that were contacted and requested to participate in this survey, 27 (~70%) of the laboratories responded. Laboratories in the United States, Ecuador, and Mexico comprise 63% of the diagnostic laboratories that were surveyed. Colombia, Panama, Brazil, Guatemala, Nicaragua, Honduras, and Costa Rica comprise the other 30% of the participating diagnostic laboratories. Nearly half (41%) of the diagnostic laboratories are associated with a university or research institution that provides services to the shrimp farming industry by cost recovery. Private, for-profit commercial laboratories (~19%) that provide service to the shrimp farming industry, and farm-owned laboratories (~26%) that are part of a shrimp farming company to which it provides almost exclusive services together comprise ~45% of the laboratories. Government-funded laboratories that serve a particular country's shrimp farming industry comprise 15% of the laboratories. Half of the laboratories responding employ less than 5 staff members (52%) while 41% employ between 6 to 10 staff members. Two laboratories employ more than 10 staff (7%).

Tests provided

The approximate number of diagnostic assays (using histological, microbiological, or molecular methods) ranged from less than 100 (7%) to greater than 10,000 (11%), with the category of 100 to 500 assays accounting for 30% of the total responses for the year 2000. Some 82% of the laboratories surveyed performed histology. Routine histology using paraffin-embedded tissues was employed by 74% of the laboratories and special stains (*i.e.* Feulgen for DNA, Giemsa for parasites, Steiner & Steiner for intracellular bacteria, etc.) were used by 60% of the laboratories.

Routine microbiology services are offered by 78% of the laboratories with routine isolation and identification of bacteria being offered by 63% of the laboratories. Methods used for bacterial identification include, classical tube methods (82%), API NFT, API 20E, or other API system (88%), the Biolog system (18%), and others such as BBL Crystal, NF/E, VITEK, and molecular methods (35%). Kirby-Bauer antibiotic sensitivity testing with commercially available disks and in-media antibiotic inhibitory concentration (MIC) assays accounted for 59% and 48% of the responses, respectively. Only 22% of the laboratories provide fungal isolation and identification services. None of the surveyed laboratories use shrimp primary cell cultures or insect and fish cell lines for shrimp virus isolation and culture.

Antibody-based (serological) techniques are used by 48% of the laboratories, with these labs performing techniques such as immunohistochemistry (40%) and ELISA tests (22%). Roughly a third of the laboratories responding use immunohistochemistry for testing histological sections for WSSV (37%), and TSV (33%). Only one laboratory indicated that they offer an antibody-based test for YHV. Of the 13 respondents that provide antibody-based tests, 11 purchase the antibodies they use from a commercial supplier (DiagXotics, Inc.), while one responded that the antibodies were made "in house."

Molecular methods (*i.e.* dot blots and *in situ* hybridization assays with gene probes, and PCR/RT-PCR) were the diagnostic methods used most frequently by the survey respondents (93%). Gene probes run as dot blots on membranes were used by 59% of the laboratories and are most frequently applied to tests for WSSV (59%), IHHNV(52%), NHP(41%), and HPV (19%). Gene probes used with *in situ* hybridization (ISH) with paraffin embedded histological sections were performed by 82% of the labs and are most frequently used for WSSV (74%), TSV (70%), IHHNV (56%), YHV (48%), NHP(41%), and HPV (24%). The gene probes used for the above procedures are generally purchased from a commercial supplier (82%), while only 15% of the respondents reported that they make the probes "in house."

Most of the laboratories contacted (74%) use gene amplification (PCR & RT-PCR) methods. The respondents indicated that they use PCR/RT-PCR to assay for WSSV (74%), YHV (48%), IHHNV (48%), TSV (44%), NHP (22%), HPV (15%), BP, MBV, rickettsia, and *Vibrio* spp. (11%). The survey indicated that most (19 of 27) of the labs purchase the oligonucleotide primers they use for PCR/RT-PCR from commercial sources. Primers for WSSV, TSV, IHHNV, and YHV are most frequently purchased by these laboratories, while only a few labs purchase primers for HPV, NHP, and other agents such as BP, MBV, rickettsia, and *Vibrio* spp., and primers for use as an internal control (e.g. for shrimp ribosomal DNA). In the Americas, PCR/RT-PCR kits are most often purchased from DiagXotics (59%), IQ-2000 (30%), and others such as Concepto Azul, Corpo Gen., and Karson Inc. (15%). The kits are most frequently used for WSSV (59%), YHV (41%), TSV (37%), IHHNV (30%), HPV (11%), and NHP (11%). The ISH kits are purchased primarily from DiagXotics (70%), and IQ-2000 (4%). The ISH are most frequently used for TSV (67%), WSSV (67%), IHHNV (44%), YHV (44%), NHP (33%), and HPV (11%).

BIOSECURITY IN PENAEID SHRIMP AQUACULTURE

"Biosecurity" has become a commonly used term in the shrimp culture industries of the Americas only in the past few years. However, the concept that it represents is, and has been, the foundation of nearly all mature and successful food animal producing industries for decades. Many producers of cattle, swine, poultry, and many aquatic species (e.g. trout, salmon, and catfish) rely on the principles of biosecurity for sustainable production (Bullis and Pruder 1999). *Biosecurity*, as it is being applied to shrimp aquaculture may be defined *as the practice of exclusion of specific pathogens from cultured aquatic stocks in broodstock facilities, hatcheries, and farms, or from entire regions or countries for the purpose of disease prevention.* Although the term "disease-free" is commonly used by marketers to describe the live shrimp products in commerce, in reality no truly "disease-free" shrimp (or any other for that matter) exist in natural or farm environments.

In the wake of the epizootics due principally to the shrimp viruses TSV and WSSV that swept through the main penaeid shrimp growing regions in the Americas, the shrimp farming industry now seems intent to utilize any of the applicable concepts of biosecurity in its farms. The application of biosecurity concepts to many of the existing types of shrimp farming, as they have been applied to poultry for example, is not something that can be accomplished easily or in short term. The industry has thousands of hectares of farms and hundreds of hatcheries (Rosenberry, 2001), few of which were designed to afford managers with much of an opportunity to totally prevent particular pathogens from being introduced and becoming established or to exclude them during normal farming activities. Nonetheless, biosecurity is a broad concept and much can be done to reduce losses due to particular pathogens by utilizing "seed stocks" that are free of the major pathogens of concern and by modifying existing farms and their management routines in order to apply biosecure concepts. Furthermore, the application of biosecurity concepts to shrimp aquaculture, will contribute significantly to making the industry much more sustainable and environmentally responsible well into the future.

Key to any effort at excluding pathogens are the following principles and tools:

1. Knowledge of diseases of concern.
2. Availability of adequate diagnostic and detection methods and services for the pathogens of concern.
3. A list of excludable diseases/pathogens of concern.
4. Control of the shrimp stocks that are farmed.
5. Adequate environmental control to prevent the introduction of pathogens of concern.
6. The use of effective management practices that ensure continuous implementation of pathogen exclusion methods and that policies are in place and practiced.
7. Disinfection and pathogen eradication plans in place to contain and eradicate disease outbreaks due to pathogens of concern.

Adequate Diagnostic and Detection Methods

The application of biosecurity by any component of the penaeid shrimp culture industry (*i.e.* a facility, a geographic region, or a country) is dependent upon the availability of sensitive,

accurate, cost effective disease diagnosis and pathogen detection methods. Those that are available from the literature, commercially in the form of kits, or from public or private diagnostic laboratories (discussed previously in this review). Highly sophisticated methods for pathogen detection in various sorts of samples are of little value to the shrimp farming industry if those methods exist only in the laboratories that developed them, but they are not readily available to an industry that could benefit from their application and use. Likewise, of little value to biosecurity programs are other diagnostic and pathogen detection methods which that may be generally available, but which are not sufficiently sensitive or accurate to meet the industry's requirements. Various manuals can serve as guides as to the available methods for disease screening and diagnosis. The OIE Diagnostic Manual for Aquatic Animal Diseases (OIE, 2000b) provides methods that have national or international approval for disease screening and diagnosis. Tables 5-6 list the OIE notifiable and listed shrimp viruses and the available diagnostic and detection methods for each.

List of Excludable Diseases/Pathogens of Concern

An important aspect of biosecurity is defining what specific diseases/pathogens or types of pathogens are to be excluded. Not all potential causes of disease in shrimp aquaculture can be excluded by the application of a biosecurity program. Shrimp have as part of their natural microbial flora and in their aquatic environment, a large and diverse population of microorganisms, some of which are facultative pathogens ready to strike when the shrimp become compromised by any number of stressors. Certain *Vibrio* spp. provide a good example of a group of organisms that live in the shrimp's environment often as part of their normal microflora inhabiting the surface of their cuticle or colonizing areas of the gut or hepatopancreas (Brock and Lightner, 1990; Fulks and Main, 1992). Some *Vibrio* spp. can become deadly pathogens in "stressed" shrimp. Hence, diseases due to "abiotic" agents (*i.e.* "stress", toxicants, environmental extremes, nutritional imbalances, etc.) or those due to opportunistic "biotic" agents that are either commonly present in the culture environment or part of the shrimp's normal microflora, are not excludable and, therefore, should not be among the listed disease agents to be excluded in a biosecurity plan. However, the management of such diseases through farm design, the use of appropriate feeds and feed application, and the quality of overall management are nonetheless essential components of successful shrimp farming.

What should be on a list of pathogens in a biosecurity program? Because not all potential causes of disease can be excluded, the development of a list of specific pathogens to be excluded is among the essential elements of an aquaculture biosecurity plan, whether it be for a single culture facility, a group of farms, a country, or an region made of several countries. However, for a list of excludable diseases/pathogens to be meaningful, certain criteria must be met. Basic information on the biology of the pathogen must be known. For example, for each listed pathogen, at least some of the key information on the host range, approximate geographic distribution, and means of transmission should be known. Generally, the disease agents listed in a biosecurity plan should be agents that: 1) are infectious and, usually, obligate pathogens; 2) are not ubiquitous or part of the shrimps' normal flora; 3) have a limited geographic and/or host range; 4) may cause economically significant losses (*i.e.* high mortality or poor culture performance); and 5) cause a disease that cannot be readily managed (*i.e.* by the use of antibiotics, disinfectants, etc.).

Examples of specific lists of excludable pathogens are available and may be helpful if referred to when formulating a pathogen list for a facility, a particular region, or a country. The U.S.

Marine Shrimp Farming Consortium's (USMSFC) publishes a list of pathogens (Table 9) that it strives to exclude from its facilities and lines of domesticated shrimp in its annual publications (Bullis, 2001). Also indicated in Table 9 are those disease agents listed by the OIE (2000a, 2000b) as "notifiable" (one asterisk) and "other significant pathogens" (two asterisks). The OIE maintains at its web site (www.oie.org) and publishes regularly an International Aquatic Animal Health Code and Diagnostic Manual. The OIE currently has nine crustacean diseases (eight of which are penaeid virus diseases) on its list of pathogens which pose a threat to international commerce, fisheries, and aquaculture of crustaceans (especially shrimp) (Table 10). Before a disease may be included in the OIE lists of notifiable and listed diseases, OIE has set criteria that must be met: 1) the etiological agent must be known, 2) reliable diagnostic(s) methods must be available, and 3) the disease must be a significant disease of local, regional, or international importance. The OIE criteria for listing are very similar to those used by the USMSFC. Pathogen lists, such as the OIE list, are useful models for setting up a biosecurity program that is based on exclusion of a list of specific pathogens and the diagnostic methods for surveillance and diagnosis.

While biosecurity has as its goal the exclusion of known pathogens for which epizootiological data is available and for which there are adequate diagnostic and detection methods, the application of biosecure practices can also reduce the likelihood of the introduction of an unknown or poorly understood pathogen. However, to be most effective, the epizootiology of a pathogen (*i.e.* its hosts, biology, and methods of transmission) that is the etiological agent of a particular disease must be sufficiently known to permit managers to understand how the pathogen is transmitted and how to prevent its entry and spread. It is impractical, if not impossible, to expect biosecurity to lead to the development of "disease free" or "pathogen-free" shrimp stocks. It is equally impractical to expect to farm such stocks in an environment where every potential pathogen is excluded.

Control of Shrimp Stocks

Perhaps the single most important principle of biosecurity is stock control. Ironically, most of the world's penaeid shrimp farming industry depends on the capture of wild postlarvae or broodstock to provide the "seed stock" used to stock farms (Argue and Warren, 1999). Introduction of diseases (pathogens) with infected live shrimp for aquaculture to new locations (*i.e.* to broodstock facilities, hatcheries, farms, groups of farms, countries, or geographic regions) was identified by recent risk assessments done in the United States (EPA, 1999) and Australia (AQUIS, 2000) as among the most likely routes by which non-indigenous shrimp viruses might be introduced. Other routes by which shrimp pathogens might be introduced to new regions were also identified in these risk assessments, including some of the routes that are ranked on a semi-log scale in Figure 1 according to their relative risk (in the author's opinion).

While some application of biosecurity principles are possible with an industry that uses wild stocks for seed production, consistency in preventing disease and pathogen introduction is problematic because of a variety of problems inherent in having laboratory testing performed.

Such problems may include limitations to the accuracy and sensitivity of the test(s) used , representative sampling and sample sizes needed for statistical confidence, and problems with getting the required samples to diagnostic laboratories, tested, and reported within what is often a

Table 9. U.S. Marine Shrimp Farming Consortium (USMSFC) year 2001 working list of "specific" and excludable pathogens of American penaeids and Asian penaeids (from Bullis, 2001)

Pathogen Type	Pathogen/Pathogen Group	Pathogen Category[2]
Viruses	* WSSV - the white spot syndrome viruses (Nimaviridae, proposed new family)	C-1
	* YHV, GAV, LOV - the Oka viruses (Roniviridae, proposed new family)	C-1
	* TSV - a picornavirus	C-1
	** BPV[1] - an occluded enteric baculovirus	C-2
	** MBV[1] - an occluded enteric baculovirus	C-2
	** BMN[1] - a non-occluded enteric baculo-like virus	C-2
	** IHHNV - a systemic parvovirus	C-1
	** SMV - an enteric parvovirus	C-1
	HPV - enteric parvoviruses	C-2
Procaryotes		
	NHP-bacterium - Alpha protobacteria	C-2
Protozoa	Microsporidians	C-2
	Haplosporidians	C-2
	Gregarines	C-3

* OIE notifiable pathogen as of May 1999.
** OIE listed pathogen as of May 1999 (OIE, 2000a; 2000b).

1 The 1995 Committee report on virus taxonomy (Murphy *et al*., 1995) removed crustacean baculoviruses from the *Baculoviridae* and assigned them to a position of unknown taxonomic position. The viruses, BP, MBV, and BMN are likely to remain in the *Baculoviridae*, while the WSSV-group is not related to the *Baculoviridae*, and it has now been proposed to represent a new family, the *Nimaviridae.*

2 Pathogen category (modified from Lotz *et al*., 1995) with C-1 pathogens defined as excludable pathogens that can potentially cause catastrophic losses in one or more American penaeid species; category 2 pathogens are serious, potentially excludable; and category 3 pathogens have minimal effects, but may be excluded from breeding centers, hatcheries, and some types of farms.

Table 10. OIE notifiable and listed penaeid shrimp diseases and their current, presently known distribution in wild and cultured stocks (modified from Lightner, 1996; Lightner and Redman, 1998; OIE, 2000b)

Virus or Virus Group	Eastern Hemisphere	Western Hemisphere
OIE Notifiable Viruses of Penaeid Shrimp:		
WSSV	wild & cultured	wild & cultured reported, but not confirmed (false positives were reported)
YHV/GAV-group	wild & cultured	
TSV	cultured	wild & cultured
OIE Listed Viruses of Penaeid Shrimp:		
IHHNV	wild & cultured	wild & cultured
BP	not reported	wild & cultured
MBV	wild & cultured	reported; not enzootic
BMN	wild & cultured	not reported
SMV	cultured	not reported

relatively short period of time between the time the wild seed stock is collected or spawned and the time by which transport and/or stocking must occur. Furthermore, the prevalence and severity of infection of significant pathogens in wild populations may be quite low, making their detection a difficult task. These factors lead frequently to false negative results when wild stocks (nauplii, larvae, PLs or broodstock) are sampled and screened using even the most sensitive molecular methods available. Hence, while more sensitive and accurate diagnostic tests are becoming available each year, no test is likely to ever be 100% accurate (OIE, 2000b). The best way to be sure of the pathogen status of any given shrimp stock is have control of the stock and to monitor it for specific pathogens over time, thus building a documented history of the particular stock as being free of specific pathogens. This is the concept of programs that develop domesticated lines of specific pathogen-free stocks.

Disease management through exclusion of specific pathogens is commonplace in modem agriculture. This concept of developing stocks that are specific pathogen free (SPF) and rearing of these stocks in regions where the specific pathogens of concern are excluded has been used in the Western Hemisphere with mixed success. The successful application of the SPF concept is, of course, dependent upon the absence of the pathogen(s) of concern in the stocks being reared (or that are present), on the availability of sensitive and accurate detection and diagnostic methods for the pathogen(s), and the presence of an effective barrier (*i.e.* facility design and geographic location,

government mandated import restrictions, etc.) to prevent the introduction of the specific pathogen(s) intended to be excluded. In situations where specific pathogens may not be excludable, the development and use of specific pathogen-resistant or SPR stocks may provide a valuable alternative to using exclusively SPF stocks.

In the Western Hemisphere, SPF stocks of *P. stylirostris* and *P. vannamei* have been developed and these are being cultured successfully in some locations (Wyban, 1992; Wyban *et al*., 1992; Carr *et al*., 1994; Pruder *et al*., 1995). The ICES Guidelines (Table 11; Sindermann, 1990) were followed for the development of these stocks. The determination of which specific pathogens the selected stocks were to be free of was based on a working list of specific, excludable pathogens (Wyban, 1992; Lotz *et al*., 1995). The most current working list for the U.S. Marine Shrimp Farming Consortium (USMSFC) includes eight viruses (WSSV, the YHV/GAV-group, TSV, IHHNV, HPV, BP, MB V, and BMN), the rickettsia-like bacterium that causes necrotizing hepatopancreatitis (NHP), and certain classes of parasitic protozoa (microsporidians, haplospordians, and gregarines) (Bullis, 2002).

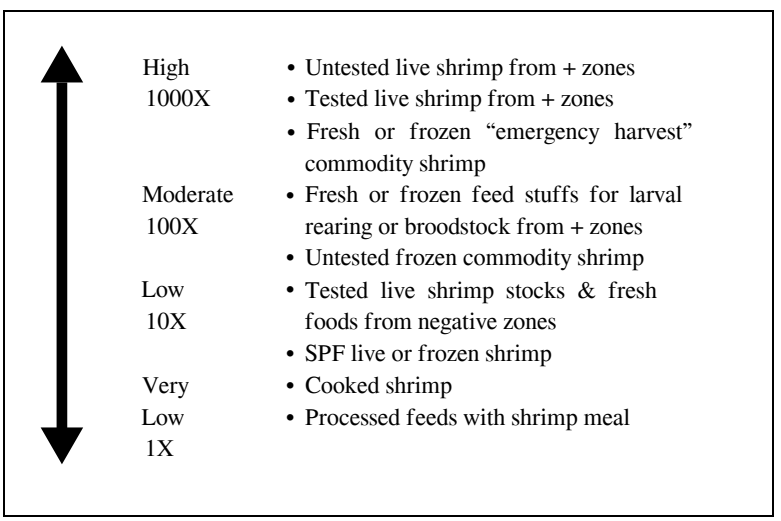

Figure 1. The relative risk presented by certain types of shrimp products, ranging from live shrimp for aquaculture, frozen shrimp for reprocessing and marketing, to processed feeds that contain shrimp meals, to the shrimp farming industry in a geographic region, a country, or a region within a country, or individual farm or hatchery

Table 11. Recommended steps in the ICES guidelines for risk reduction in aquatic species introductions (modified from Sindermann and Lightner, 1988)

Original ICES Guidelines	Adapted to SPF shrimp development
1. Conduct comprehensive disease study in native habitat	1. Identify stock of interest (*i.e.* cultured or wild)
2. Transfer {founder stock} system in recipient area	2. Evaluate stock's health/disease history
3. Maintain and study closed system population.	3. Acquire and test samples for specific listed pathogens (SLPs) and pests
4. Develop broodstock in closed system	4. Import and quarantine founder (F_0) population; monitor F_0 stock
5. Grow isolated F individuals; destroy original introductions	5. Produce F_1 generation from F_0 stock
6. Introduce small lots to natural waters - continue disease study	6. Culture F_1 stock through critical stage(s); monitor general health and test for SLPs
	7. If SLPs, pests, other significant pathologies are not detected, F-1 stock may be defined as SPF and released from quarantine

SPF stocks developed by the USMSFC were developed in the spirit of the ICES Guidelines (Table 11; Figure 2). To begin the process, each "SPF candidate population" of wild or cultured shrimp stocks of interest was identified. Samples of the stock were taken and tested using appropriate diagnostic and pathogen detection methods for the specific pathogens of concern. If none were found, a founder population (F_0) of the "candidate SPF" stock was acquired and reared in primary quarantine. During primary quarantine, the F_0 stock was monitored for signs of disease, sampled, and tested periodically for specific pathogens. If any pathogens of concern were detected, the stock was destroyed. Those stocks that tested negative for pathogens of concern through primary quarantine (which ran from 30 days to as much as 1 year for some stocks) were moved to a separate secondary quarantine facility for maturation, selection, mating, and production of a second (F_1) generation. The F_1 stocks were maintained in quarantine for further testing for specific pathogens of concern. Those that tested negative were designated as SPF and used to produce domesticated lines of SPF and "high health" (Wyban *et al*., 1992; Pruder *et al*., 1995). The SPF and high health stocks of *P. vannamei* were used successfully in U.S. shrimp farms in 1993, 1994, and since 1997 resulted in more than double the production per crop that had been previously obtained at the same farms in previous years when the farms cultured non selected lines of *P. vannamei,* which in previous crops, had been persistently affected by "runt deformity syndrome" (RDS) due to chronic infection by IHHNV, or affected by TSV and WSSV (Brock and Main, 1994; Pruder *et al*., 1995; Lightner, 1996a, 1996b; Bullis and Pruder 1999).

Stock control is a critical component of biosecurity. As long as the industry in the Americas remains dependent on wild stocks, it cannot expect to be consistently successful in excluding pathogens of concern in the wild seed stocks that it relies on. The use of wild broodstock, and especially wild postlarvae, leaves the farms that rely on this source of seed stock particularly vulnerable to the introduction of pathogens of concern. While numerous methods have been incorporated into the operational design and management of shrimp farms previously affected by TSV and WSSV to eradicate them and to insure that they are not reintroduced, none can be expected to provide much protection against crop losses in farms that use seed stock derived from

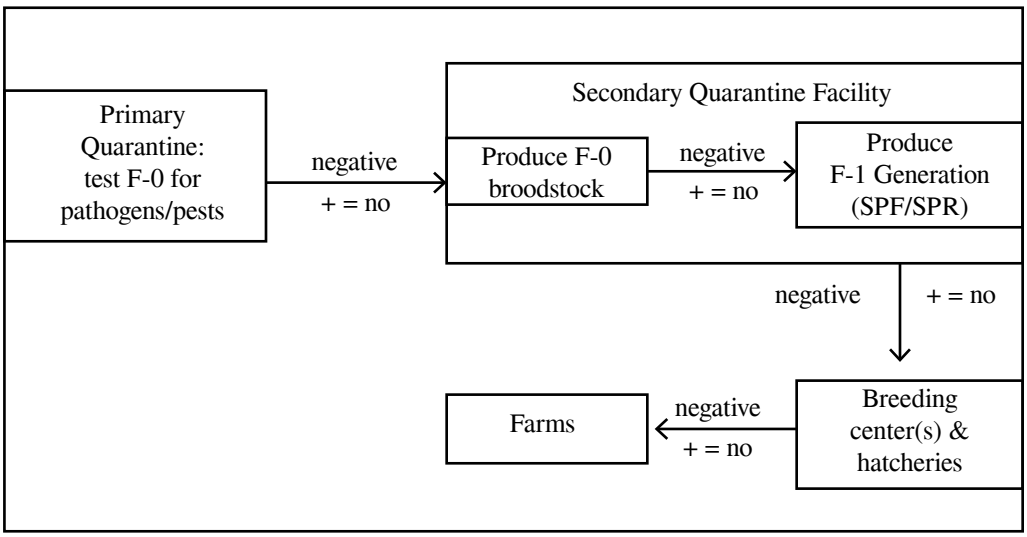

Figure 2. Schematic diagram of the steps followed by the U.S. Marine Shrimp Farming Consortium (USMSFC) in developing specific pathogen-free (SPF) breeding lines

wild stock sources. The use of only domesticated shrimp stocks that have a known history of being free of pathogens of concern can help to mitigate this risk. However, a SPF history comes only from a long-term captive breeding and disease surveillance program at a facility that has a fully functional and effective biosecurity plan.

ENVIRONMENTAL CONTROL AND BEST MANAGEMENT PRACTICES

A variety of environmental and best management practice strategies have been attempted for the control of viral diseases in penaeid shrimp aquaculture. These strategies range from the use of improved culture practices (*i.e.* where sources of virus contamination are reduced or eliminated, source water is treated, filtered, and aged to remove potential vectors, culture ponds fallowed and treated between crops, routine sanitation practices are improved, stocking densities are reduced, etc.) to stocking "specific pathogen-free" (SPF) or "specific pathogen resistant" (SPR) species or stocks. Because these topics are beyond the scope of the present review, the author refers the reader to other papers in which this topic has been thoroughly reviewed (Bullis and Pruder, 1999; Lee, in press; Takashima *et al.*, 2001).

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An Overview of PCR Techniques for Shrimp Disease Diagnosis in Asia, with Emphasis on Thailand

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ABSTRACT

Asia leads the world in cultivated shrimp production with export earnings in the order of billions of US dollars per year. In spite of this success, annual production decreased in the late nineties because of widespread epizootics caused by new viral pathogens. Although, these viruses were no cause for alarm to human health authorities, they were economically crippling for Asian shrimp farmers. In Thailand, shrimp production trends have mirrored those in the rest of Asia, except that recovery from the viral epizootics has been somewhat better than it has for most of its close neighbors. Our work in Thailand has focused on the characterization of the causative viruses and on the development of rapid diagnostic probes for them. Similar work has been done elsewhere. The aim of the work has been to develop effective control measures to help shrimp farmers. We are engaged in similar work on bacteria and parasites. The major viruses of concern (in our estimated order of economic impact for Thailand) are white-spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV) and infectious hypodermal and haematopoeitic virus (IHHNV). We have also prepared probes for *Vibrio parahaemolyticus* and for a microsporidian parasite, *Agmasoma penaei.* These highly specific and sensitive tools for detection are already helping shrimp farmers and we hope that new technological advances will make them practicable in the field. At the moment, however, the most rapid test is the polymerase chain reaction (PCR) test, which takes approximately 3 hours to complete. This review covers important Asian shrimp diseases for which PCR tests are currently available.

INTRODUCTION

Asia has always led world production of cultivated shrimp with a market value of billions of US dollars per year. Thailand alone has been the world's leading producer since 1992 with its export earnings alone reaching more than one billion US dollars per year. However, in Thailand in 1995, largely due to yellow-head virus (YHV), production decreased by about 5,000 metric tons (equal to approximately 40 million US dollars in lost export revenue) (Flegel *et al*., 1995b). In 1996 and 1997, another virus called white-spot syndrome virus (WSSV) was even more disastrous, with cumulative lost export revenue estimated at approximately 1 billion US dollars (Flegel and Alday-Sanz, 1998; Flegel, 1997). After 1997, Thai production began to recover, reaching the previous highest production of 250,000 metric tons again in 1999. The rest of Asia did not fare so well. For example, WSSV outbreaks in China began in 1993, reducing export production from the 1992 high of 115,000 metric tons to 35,000 metric tons. Recovery has been slow, with production reaching only 70,000 metric tons by 1999.

These examples serve to illustrate how serious disease losses can be in the shrimp aquaculture industry. The perilous position of the shrimp farmer and the shrimp industry can be greatly improved by the implementation of relevant strategies which include programs for improved farmer cooperation and technological changes. These strategies could lead to a long term, stable shrimp industry with little negative environmental impact. Biotechnological research can make substantial contributions towards maintaining achieving this goal but it is essential that government and industry provide continuous support for the infrastructure and training required to maintain the relevant capability.

This review covers steps in the development of DNA probes and PCR technology for detection of shrimp pathogens. Much work described has been done in Thailand and it has been reviewed in a broader context elsewhere (Flegel, 1997). Where appropriate, similar work done elsewhere will be included. While focusing on these probes, one should not forget that the probes play only one small part in the overall strategy to fight against disease. They are not an answer in themselves but must be used properly in the overall context of a shrimp health program involving such topics as environmental safety, nutrition, and genetics, to name only three.

This review will cover the development of DNA diagnostic probes for white-spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV) and infectious hypodermal and haemotopoeitic parvovirus (IHHNV). Also briefly discussed will be probes developed in Thailand for *Vibrio parahaemolyticus* and for the microsporidian parasite, *Agmasoma penaei,* and probes of others for Taura syndrome virus (TSV), spawner mortality virus (SMV) and a mycoplasma. In terms of losses to the Asian shrimp industry WSSV, YHV, HPV and MBV are undoubtedly the most important (in decreasing order). Losses from the virus IHHNV and from bacteria and microsporidians are less clearly evident.

MONODON BACULOVIRUS (MBV)

We were quite alarmed when we saw this virus (Figs. 1-3) in Thailand for the first time in 1990 (Fegan *et al*., 1991), because it had been implicated in the collapse of the shrimp industry in Taiwan in the mid 1980's (Lin, 1989). However, we soon found out that it did not cause shrimp mortality so long as rearing conditions were good. This was in spite of the fact that some of the infected shrimp larvae had very large numbers of viral inclusion bodies. In other words, they survive high levels of virus production with no ill effects and no visible resistance response. The opinion that MBV did not cause mortality was later expressed in Taiwan as well (Liao *et al*., 1992).

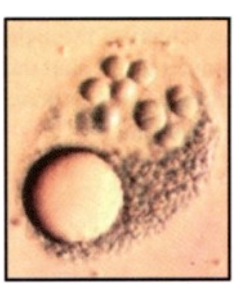


Figure 1. Squash mount of a larval hepatopancreatic cell showing several polygonal viral inclusion bodies in the enlarged nucleus. The inclusions are composed of a protein matrix called polyhedrin which contains embedded viral particles. Because these protein particles enclose or protect viral particles, they are sometimes called occlusion bodies. They are released in the shrimp feces and ingested by other larvae so that the infection is spread horizontally

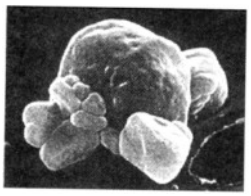
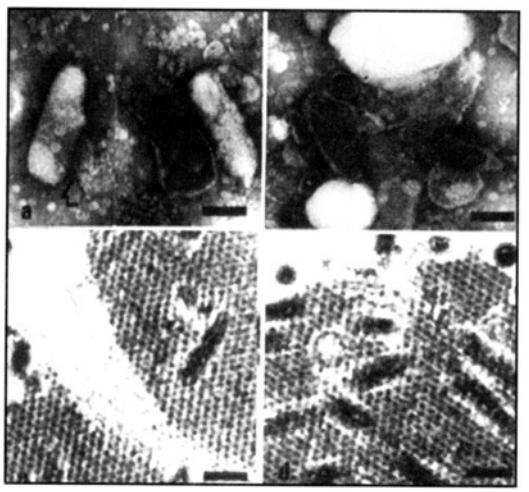


Figure 2. Scanning election micrograph of MBV inclusion bodies purified from shrimp larvae by differential centrifugation of tissue homogenates. Such particles can be used for subsequent viral purification and nucleic acid extraction

Figure 3. Transmission election micrographs of MBV. The upper left photograph shows two virions with intact envelopes on the left and right and one with a fractured envelope revealing the inner nucleocapsid. The lower right photograph shows a section of an inclusion body with rod shaped virions embedded in the polyhedrin matrix



Even though we knew that MBV was not a serious pathogen for the black tiger prawn, we still wanted to eliminate it from the farming system because we did not believe that the shrimp could carry such heavy viral infections without paying some price. Indeed, we have done further work showing that the mean length of MBV infected shrimp is significantly shorter than uninfected shrimp from the same pond (Fig. 4) (Flegel *et al.*, 2001), although this difference was not easily detected until late in the cultivation cycle. These results support findings from studies in the Philippines indicated that MBV infections could slow growth in intensive cultures. We also suspect that poor rearing conditions could lead to a flare-up of the virus followed by secondary bacterial infections resulting in shrimp death. In fact, it turned out the that virus could be eliminated from the rearing system by a combination of washing eggs and/or early naupliar stages with clean sea water, separate rearing of single-spawn larval batches and discard of occasionally infected batches of larvae or postlarvae (PL).

The experience with MBV had important spin-offs in terms of cooperation with other scientists working on shrimp diseases. It started our contact with Australian scientists through cooperation with Dr. Joan Vickers at the University of Queensland in what comprised our start on viral gene cloning work and DNA diagnostic probe development. It also started our interactions with Dr. S.N. Chen in Taiwan, Dr. D.V. Lightner in Arizona and Dr. J.A. Brock in Hawaii.

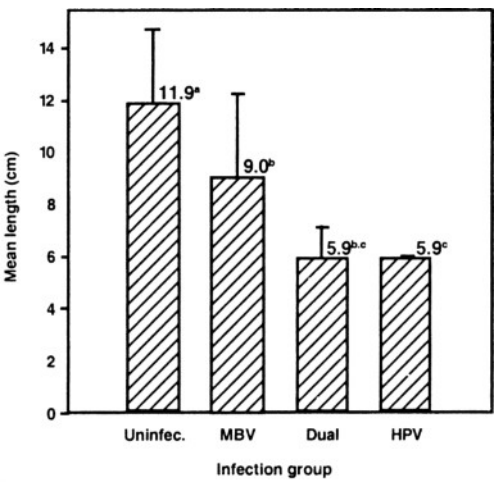


Figure 4. Comparison of infection status with length for uninfected shrimp and shrimp infected with MBV, HPV, or both MBV and HPV (dual). The shortest shrimp were those with HPV or dual MBV/HPV infections and the longest were those with no infection. Bars marked with different letters (a,b,c) indicate means that are significantly different (P<0.05) (Flegel *et al*., 2001)

Two mixed primers for the detection of MBV by PCR amplification have been published (Chang *et al*., 1993). These were designed based on conserved regions of the insect polyhedrin genes and they give rise to fragment of approximately 600 bp with MBV. These primers were also used to prepare the 600 bp fragment for use as a DNA probe for dot blot hybridization and for *in situ* hybridization. The primers are:

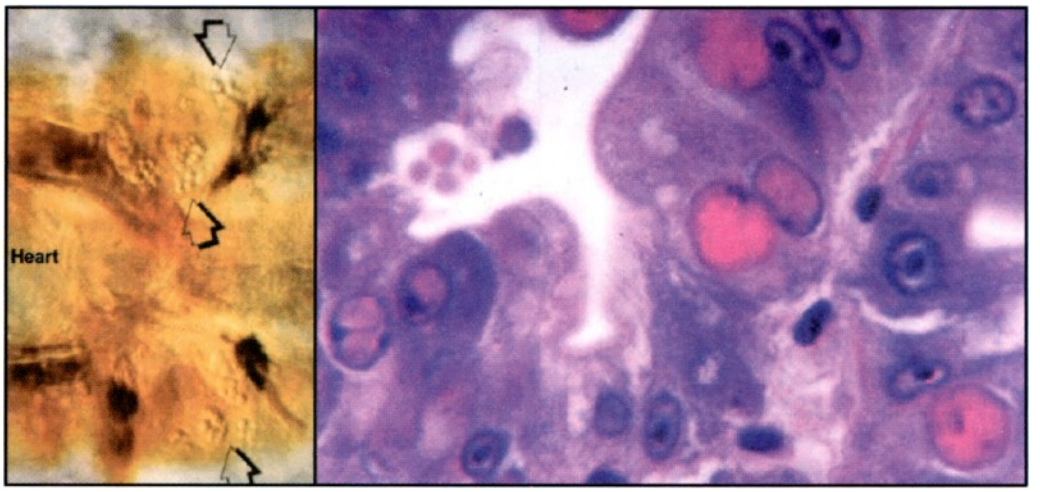
5'-AC(CT) TA(CT) GTG TAC GAC AAC AAA TA(CT) TAC AAA-3'
5'-GG(TC) GCG TGTG) GG(TC) GCA AA(CT) TC(CT) TT(TA) AC(TC) TT(GA) AA-3'

A better nested PCR process for MBV detection has been published from Australia (Belcher and Young, 1998). The primers were derived from a cloned fragment of DNA prepared from purified MBV. The two PCR products were 533 and 361 bp and sensitivity was very high at 0.01 fg MBV DNA in mixtures containing whole postlarval DNA. The primer sequences are:

1st round sense:	5'	-CGA	TTC	CAT	ATC	GGC	CGA	ATA-	3'
1st round antisense:	5'	-TTG	GCA	TGC	ACT	CCC	TGA	GAT-	3'
2nd round sense:	5'	-TCC	AAT	CGC	GTC	TGC	GAT	ACT-	3'
2nd round antisense:	5'	-CGC	TAA	TGG	GGC	ACA	AGT	CTC-	3'

Our work with MBV continues, albeit at a lower level of intensity. We have used the Belcher and Young (1998) probe for successful detection of MBV from shrimp feces by dot-blot DNA hybridization and PCR amplification. This would allow for non-destructive testing of broodstock and pond reared shrimp. More work is needed on the MBV genome particularly with respect to comparative analysis with the insect NPV for which much more information is currently available. Such studies should give some insight into the interaction between MBV and its shrimp host.

Since MBV is a DNA virus like WSSV and HPV (see below for details on these two viruses), it should be possible to devise a multiplex PCR method that would be capable of detecting any combination of these viruses in DNA extracts from PL. Since PL in Thailand are already regularly tested for WSSV by PCR, it would seem a worthwhile goal to assay for all of these viruses in a single PCR reaction.



Additional figure. Photomicrograph on the left shows MBV occlusion bodies (arrows) as viewed directly through the cuticle of an early PL specimen using the light microscope with a 40x objective. The photomicrograph on the right shows early to late stages of MBV infection in an H&E stained tissue section of the hepatopancreas (HP). Nuclei become enlarged with an acidophilic (pink) center with nucleoli and chromatin condensed along the nuclear membrane. At the final stage the very enlarged nuclei contain acidophilic, paracrystalline protein inclusions (occlusion bodies). In this photomicrograph, 4 occlusion bodies can be seen free in the lumen of the HP where they have been discharged from a lysed cell

YELLOW-HEAD VIRUS (YHV)

Our work on MBV was interrupted by the arrival of the first really serious viral pathogen of shrimp in Thailand in 1992. In retrospect, we know that this virus first began to cause problems in Thailand in 1990 (Limsuwan, 1991), but it was not discovered as a new pathogen until 1992 (Boonyaratpalin *et al*, 1993; Chantanachookin *et al*., 1993). The virus was named from the gross signs of disease which included a yellowish cephalothorax and very pale overall coloration of moribund, infected shrimp (Fig. 5). Histologically, it can be recognized by densely basophilic inclusions, particularly in the gills by rapid staining (Flegel *et al*., 1997a) (Fig. 6) and the haemolymph (Nash *et al*., 1995) (Fig. 7). Research on YHV in Thailand has been reviewed (Flegel *et al*., 1997a; Flegel *et al*., 1995b) along with current practices for diagnosis, prevention and control.

YHV was first thought to be a baculovirus but we discovered during purification and characterization that it had curious morphology (Figs. 8 and 9) and that it was an RNA virus (Wongteerasupaya *et al*., 1995a). DNA diagnostic probes were prepared by cDNA preparation and cloning, although these currently work best in RT-PCR assays (Wongteerasupaya *et al*., 1997) rather than *in situ* hybridization (Fig. 8), probably because of the instability of the viral RNA. Even with the PCR assay, samples must be processed quickly, since storage at -80°C does not prevent deterioration of the RNA.

Figure 5. Gross signs of yellow-head infection are seen here in the 3 shrimp on the right. They are generally bleached in color with a yellowish discoloration of the cephalothorax ("head") region when compared to shrimp of normal appearance on the left

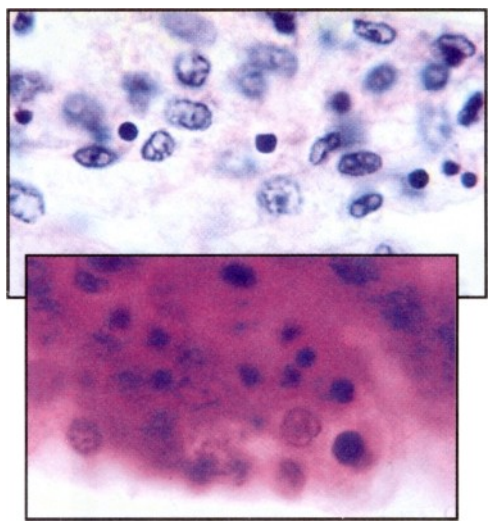


Figure 6. Gills of YHV infected shrimp stained with H&E in normal paraffin sections (upper plate) and in rapidly fixed and stained (3 hr) whole mounts (lower plate). The densely stained purple (basophilic) inclusions contrast sharply with the normal nuclei which are larger and show scattered staining of the chromatin. These gill inclusions are evident only in moribund shrimp and consequently do not have diagnostic predictive value

The PCR amplicon sequence and the primers for YHV detection in infected shrimp by RT-PCR are as follows (Wongteerasupaya *et al*., 1997):

CCG CTA ATT TCA AAA ACT ACG ACA GAA ACA CCG GCA TGT CCT GTT CTC TCA
CTG AAT TCC AGC TCT CTC TCT CTC ACA TCC TCT ACC GTT CTG AAG CAC AGC
GTA CTC CTG ACG ACT TCC TCG ACA TAA CAC CTT

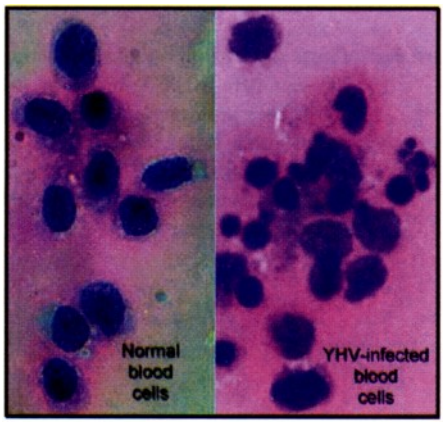


Figure 7. Haemolymph from normal and YHV infected shrimp. The disintegration of the nuclei is clearly evident in the YHV infected shrimp. These can be seen in early stages of infection, but not later when the haemocyte population has been depleted by the virus. In addition, such disintegration can be found with some bacterial infections

Figure 8. Transmission electron micrograph of YHV-infected shrimp tissue showing the unusual filamentous nucleocapsid precursors (on the left) and mature, rod-shaped, enveloped virions (on the right)

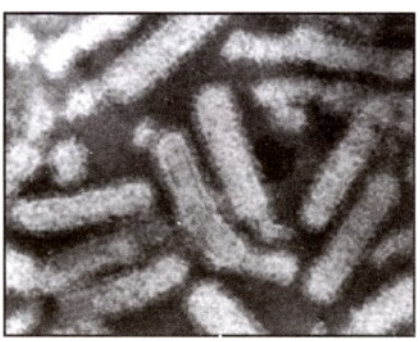


Figure 9. Transmission electron micrograph of negatively stained purified virions of YHV. Note that the virus particles are enveloped and that the envelope has a halo of appendages characteristic of some RNA viruses

This fragment can also be labeled and used for dot blot or *in situ* hybridization assays. The problem with these techniques is that the viral RNA is very labile and must be protected during specimen preparation. For *in situ* hybridization using fixed tissues, it is important not to use Davidson's fixative which is acidic and will destroy the RNA. More successful *in situ* hybridization results using RNA-friendly fixation of shrimp tissues has been reported by Tang and Lightner (1999). Briefly this method uses a formula where the acetic acid in Davidson's fixative is replaced with additional formalin. After overnight fixation, the tissues (without cuticle, which cannot be cut unless decalcified) are quickly embedded in paraffin after which they can be stored indefinitely before *in situ* hybridization assays are carried out. However, once rehydrated, the specimen sections will be extremely vulnerable to attack by RNase and all precautions must be taken to protect the specimens. We have used neutral buffered formalin and Davidson's fixative with the acetic acid

replaced by distilled water as alternative fixatives to Davidson's and these appear to work equally well. It has also been proposed that normal Davidson's fixative may be used, so long as the fixation is not longer than overnight and followed immediately by dehydration and embedding in paraffin.

The Tang and Lightner (1999) probe used for *in situ* hybridization was a 1051 bp dioxygenin labeled probe derived from a 1061 bp YHV cDNA clone and produced from that clone template using PCR with the following primers:

Sense:	5'	-ACA	TCT	GTC	CAG	AAG	GCG	TC - 3'
Antisense	5'	-GGG	GGT	GTA	GAG	GGA	GAG	AG - 3'

They also gave primers derived from the same cDNA fragment for detection of a 273 bp YHV specific amplicon by RT-PCR:

Sense:	5'	-CAA	GAT	CTC	ACG	GCA	ACT	CA-3'
Antisense:	5'	-CGA	CGA	GAG	TGT	TAG	GAG	G-3'

In addition to these reagents, Cowley *et al.* (2000a) have published primer sequences that can be used for detection of both YHV and the related Australian lymphoid organ virus (LOV) (Spann *et al*., 1995) and gill associated virus (GAV) (Spann *et al*., 1998). These primers were designed from a 781 bp GAV cDNA clone to give a 618 bp RT-PCR product and are:

Sense:	5'	-AAC	TTT	GCC	ATC	CTC	GTC AC-3'
Antisense:	5'	-TGG	ATG	TTG	TGT	GTT	CTC AAC-3'

Together with Dr. Walker's group from CSIRO Australia, sequencing and comparison of the 618 bp RT-PCR fragments obtained using these primers with YHV, GAV and LOV has shown that all are closely related single standed, positive sense RNA viruses that will likely be the first invertebrate representatives from the Order *Nidovirales* (Cowley *et al*., 2000b). LOV and GAV shared approximately 95% DNA sequence homology and 100% amino acid homology establishing that they are the same virus strain, while GAV and YHV shared approximately 85% DNA sequence homology and 96% amino acid homology indicating that they are different strains (Cowley *et al*., 1999). An excellent commercial kit is available from Intelligene of Taiwan that gives differential and graded RT-PCR detection for GAV and YHV.

Dr. Walker's group (CSIRO, Brisbane) has recently found a third, apparently non-pathogenic YHV variant in Viet Nam and Thailand that is closer in sequence to GAV than the original Thai YHV. Using the Intelligene kit, the new YHV strain gives the same PCR band as Australian GAV. These RT-PCR probes are also useful for examining suspected carries of YHV and testing whether they can transfer the virus to cultivated shrimp. The results of such studies will have an important impact on disease control programs for shrimp farmers. Dr. Walker's group has developed an excellent preservative solution for field samples. It contains 80% ethanol, 20% glycerol and 0.25% mercapto-ethanol (prepared by mixing 80 ml absolute ethanol, 20 ml pure glycerol and 0.25 ml of mercapto-ethanol). Samples are crushed or homogenized in this at 1 part sample to 10 parts preservative and survive storage for a reasonable length of time at room temperature. At the laboratory, supernatant preservative is removed before nuclei acid extraction (RNA or DNA) from tissues in the usual manner for use as a PCR template.

WHITE-SPOT SYNDROME VIRUS (WSSV)

Historically, this was the second viral infection to seriously affect Thai shrimp farmers. We have recently reviewed the studies and control methods for this virus in Thailand (Flegel et al., 1997a). Infections with it usually give gross signs of white inclusions of various sizes embedded in the carapace at the late stages of infection (Fig. 10). These characteristic gross signs of infection were first reported from an outbreak which occurred in *P. japonicus* in Japan in 1993. The causative agent was a new bacilliform virus (Takahashi *et al*., 1994). In the same interval viral infections with similar gross signs were seen in *P. japonicus. P. monodon* and *P. penicillatus* in Taiwan and China (Chou *et al*., 1995). With hindsight, we now know that in Thailand the virus was first seen in laboratory reared *P. monodon* in late 1993 (Wongteerasupaya *et al*., 1995b). Now, the virus is called white spot syndrome virus (WSSV) by general consensus (Lightner, 1996; Lightner and Redman, 1998). However, it was not found in Thai farmed shrimp until late 1994, when mass mortalities began to be reported with characteristic gross signs of WSSV infection (Wongteerasupaya *et al*., 1996).

We originally called WSSV a baculovirus based on its cylindrical morphology and histological lesions that resembled those of "non-occluded" baculoviruses (Wongteerasupaya *et al*., 1995b) and in one publication, we were actually requested by the reviewers to call it PmNOBII for "*Penaeus monodon* non-occluded baculovirus II" (Wongteerasupaya *et al*., 1996). In the end, this turned out to be a mistake. We now know that WSSV is a tailed, rod-shaped, double stranded DNA virus with a very large circular genome in the order of 300 kpb that is available at GenBank (Lightner, 1996; van Hulten *et al*., 2001; Wongteerasupaya *et al*., 1995b). Since the genome has no significant homology to any known virus, a new viral family (*Nimaviridae*) and genus (*Whispovirus*) have been proposed for it (van Hulten *et al*., 2001).

Figure 10. Gross signs of WSSV infection. White inclusions in the cuticle of moribund shrimp are indication of infection with this virus. To confirm the infection, histological examination should be made (See Fig. 11)

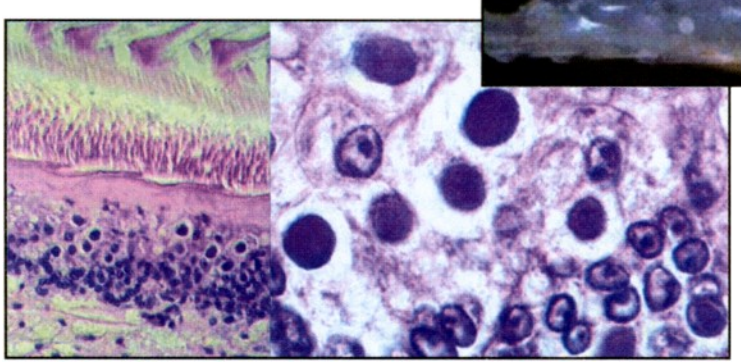


Figure 11. Histopathology of WSSV. The low magnification micrograph on the left shows many characteristic inclusion of WSSV under the cuticle of the gut epithelium. The high magnification on the right clearly shows the hypertrophied nuclei. Note the nucleus in the lower right comer with a densely red stained center (acidophilic) surrounded by a clear space and then a ring of purple (basophilic) chromatin. This is a Cowdry A-type inclusion characteristic of nuclei in the early stages of infection

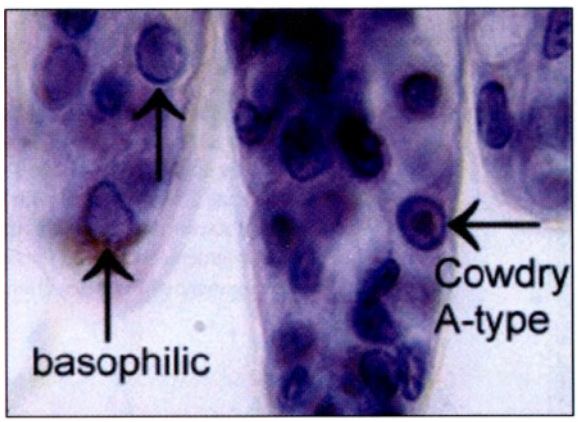


Figure 12. Rapidly stained whole gill fragment showing typical WSSV histopathology. This technique can also be used with sub-cuticular epithelial tissue for rapid histological confirmation of WSSV infections. Note the Cowdry A-type inclusion in the nucleus on the far right. The other nuclei, at later stages of infection have more basophilic centers

On the basis of gross signs of disease, histopathology with the light (Figs. 11 and 12) and electron microscopes (Fig. 13), and DNA characteristics (Fig. 14), it soon became obvious that these infections could be ascribed to the same virus or closely related forms of it (Chou *et al*., 1995; Durand *et al*., 1996; Kimura *et al*., 1996; Wang *et al*., 1995; Wongteerasupaya *et al*., 1995b). This contention was further supported by *in situ* DNA hybridization tests with white-spot syndrome, cultivated shrimp of various species from several Asian countries (Wongteerasupaya *et al*., 1996) (Fig. 15).

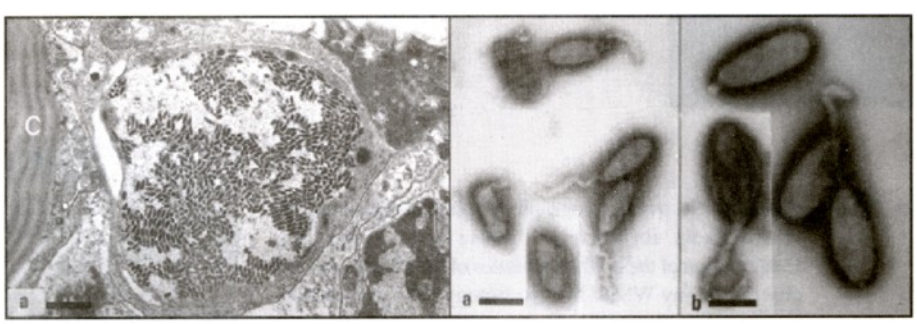


Figure 13. Transmission electron microscopy of WSSV. On the left is a low magnification view of a WSSV infected nucleus from gill tissue showing large numbers of rod shaped virions. On the right is a view of negatively stained enveloped virions showing unusual appendages and somewhat variable morphology

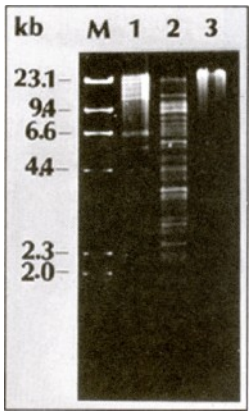


Figure 14. Polyacrylamide gel electrophoresis of nucleic acid from WSSV. Lane M contains a DNA marker and Lane 3 contains the undigested nucleic acid. The nucleic acid in Lane 1 was digested with BamHI while that in Lane 2 was digested with EcoRI. The EcoRI digest was cloned and screened for fragments that was specific for WSSV. The selected fragments were used for *in situ* hybridization assays and for development of PCR detection assays

It is extremely important to understand that diagnosis for WSSV infection cannot be based on the gross signs of white inclusions in the cuticle. A recent report by Wang *et al.* (2000) has shown that bacterial infections of the cuticle can also be associated with the formation of white inclusions, in the absence of WSSV infection (Fig. 15). Since the management response to bacterial and viral infections is fundamentally different, it is always necessary to confirm whether shrimp with such gross signs also show the histopathology characteristic of WSSV. This can be done by microscopic examination of whole gill fragments that have been rapidly fixed and stained in a simple, inexpensive process that takes only 3 hours.

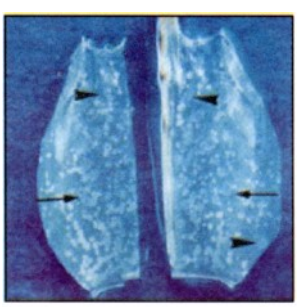


Figure 15a. Gross appearance of the carapace of shrimp showing bacterial white spot syndrome. These spots cannot be easily distinguished from those caused by WSSV, so histological examination is always required in the confirmation of WSSV infections (from Wang *et al.*, 2000)

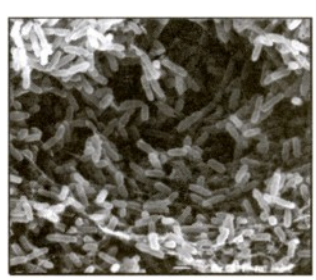


Figure 15b. Scanning electron micrograph of bacteria colonizing the white spots in bacterial white spot syndrome. Examination of the epithelial tissues of the shrimp showed absence of any WSSV histopathology. The shrimp were also negative for WSSV by nested PCR assay (Wang *et al.*, 2000)

We developed DNA hybridization probes for WSSV (Wongteerasupaya *et al*., 1996) (Fig. 16) and soon thereafter the primers for detection of WSSV by PCR, although the primer sequence was not published (Kanchanaphum *et al*., 1998) but they are available commercially from the Shrimp Biotechnology Business Unit, National Center for Genetic Engineering and Biotechnology, Bangkok. These primers are widely used in Thailand for screening broodstock and PL in an attempt to stem the spread of the WSSV and restore production to former levels. The rapid implementation of this PCR screening system together with other appropriate management (Chanratchakool and Limsuwan, 1998; Flegel *et al*., 1997a; Withyachumnamkul, 1999) has probably rescued the Thai shrimp industry from a disaster similar to that which occurred in China in 1993. By our reckoning, the preventative measures have probably saved the country in the order of 1 billion US$ per year in export earnings since 1995. Again we are cooperating with Dr. Walker's group in Australia and with scientists in Taiwan and Japan in the analysis of viral DNA sequences from various sources, in order to understand the relationship amongst the epizootics that are occurring throughout Asia and now the Americas.

Another use of the PCR test has been to identify and monitor the transfer of WSSV from reservoir hosts to shrimp. In these studies, the most surprising feature has been the wide range of potential hosts. WSSV infects not only several species of penaeid shrimp including those cultivated in the western hemisphere (Lu *et al*., 1997), but apparently also a wide range of other decapods, including crabs and more distantly related crustaceans such as copepods and perhaps even aquatic insect larvae (Lo *et al*., 1996a; Lo *et al*., 1996b). For shrimp farmers, it is extremely important to establish whether these non-cultivated crustaceans are *bona fide* reservoirs of the virus that can transmit it to cultivated shrimp. Studies in Thailand (Supamattaya *et al*., 1998) have proven that the swimming crab, *Portunus pelagicus*, and the mud crab, *Scylla serrata,* can be infected with WSSV by injection or feeding, and we have shown by time-course PCR assay, histopathology, *in situ* DNA hybridization that *P. pelagicus,* and the mangrove crab can transmit the virus back to shrimp within a few days via water (Kanchanaphum *et al*., 1998) (Fig. 17).

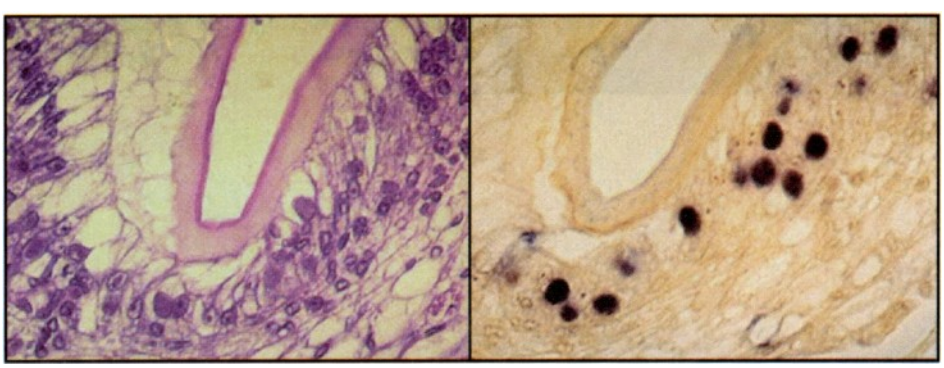


Figure 16. *In situ* hybridization detection of WSSV. The panel on the left shows H&E staining of WSSV infected tissue and that on the right shows a serial section of the same tissue assayed by *in situ* hybridization. The dark cells indicate positive hybridization

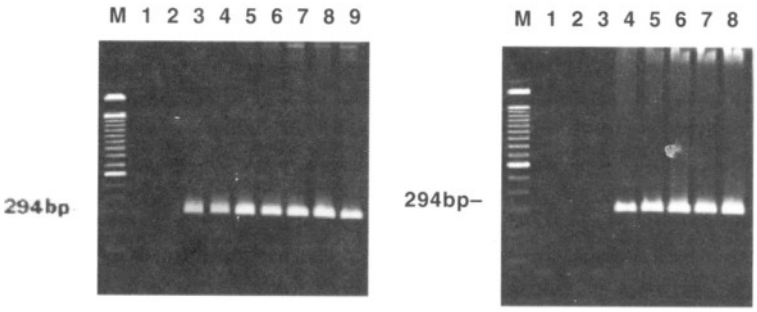


Figure 17. Examples of PCR amplification tests for WSSV in a reservoir host. Here on the left is seen an agarose gel with PCR products derived from samples of the crab Sesarma with a strong product band seen in lane 4 at 36 h after injection of the virus. The gel on the left shows the appearance of positive PCR bands for the presence of WSSV in the haemolymph of shrimp cohabitants with the infected crabs. The virus was transferred and became evident in the haemolymph by 24 h. Lane 1 = molecular marker; lanes 1-9 = products from haemolymph at 0 h (lane 1) and every 12 hours thereafter

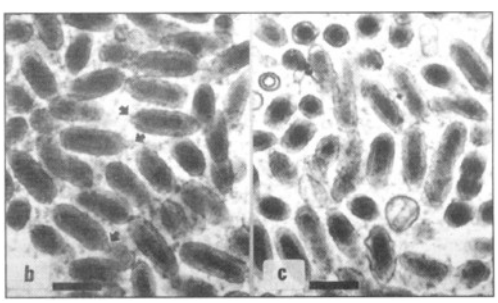


Figure 18. Transmission electron micrograph of intranuclear WSSV virions at high magnification in a tissue section of an infected shrimp specimen

HEPATOPANCREATIC PARVOVIRUS (HPV)

We have been interested in this virus for some years (Flegel *et al*., 1992b; Flegel *et al*., 1995a) but it has been hard to get information. Our preliminary data (Flegel *et al*., 1995a) suggested to us that this virus was lethal to shrimp larvae during the interval of the first month after stocking. However, our most recent results (Flegel *et al*., 1999) suggest that there is a strong statistical correlation between HPV infection and small size, suggesting that most HPV-infected shrimp simply grow very slowly and stop growing at around 6 cm in length (Figs. 4 and 19), Thus, when the shrimp are sampled by cast net, these non-growers escape and are usually counted as non-survivors. Functionally speaking for the farmer, this is almost equivalent to death since shrimp 6 cm in length weigh only about 5 g (200 pieces per kg) and have so little market value that they do not cover the cost of rearing. If HPV infected shrimp constitute a substantial part of a pond population, the resulting crop may be a financial loss. On the basis of our recent results, we are recommending that PL batches with moderate to high prevalence of HPV infections be rejected for stocking by shrimp farmers. However, there is no indication that HPV infections spread horizontally in growout ponds, so it may be acceptable to use PL batches with a low prevalence of HPV, if no uninfected batches are available and if the projected loss would be economically acceptable.

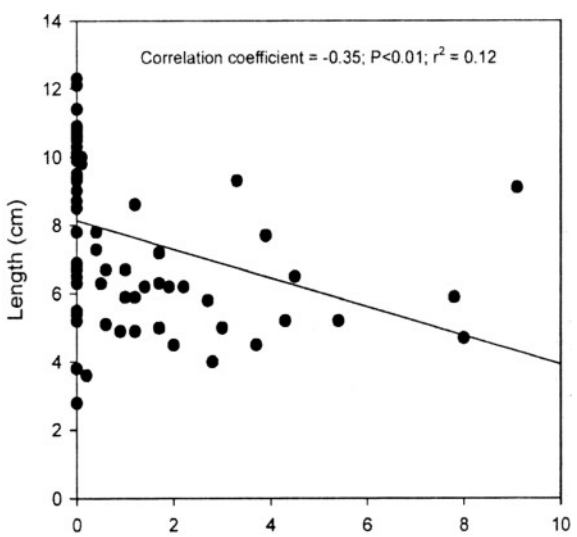


Figure 19. Severity of HPV infection versus length for shrimp from a farming system in Southern Thailand (Flegel *et al*., 1999). Please also refer back to Fig. 4, where shrimp size is shown according to infection group. There, HPV infected shrimp comprise the groups with the smallest sizes as either a single or dual infection with MBV

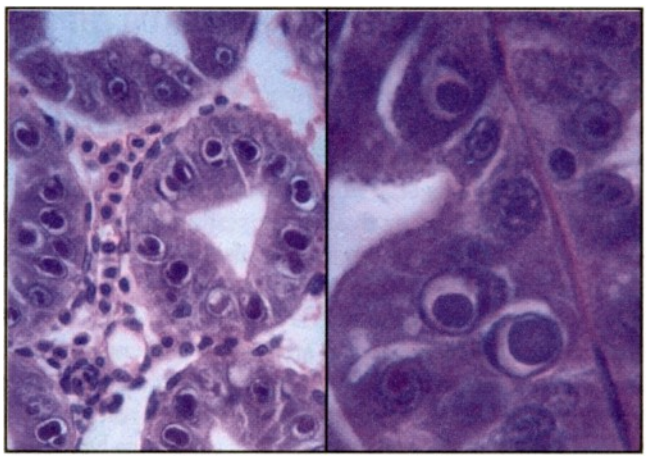


Figure 20. Histopathology of HPV infections. The panel on the left shows a low magnification of hepatopancreatic tubule epithelial cells infected with HPV. It produces densely purple stained (basophilic) intranuclear inclusions. On the right, these inclusions are shown at high magnification together with crescent shaped nucleoli which they have pushed to one side of the nucleus

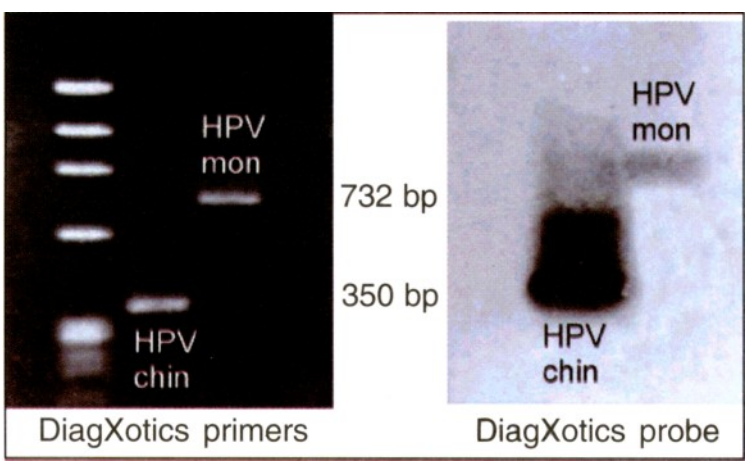


Figure 21. PCR assay of Thai HPV using primers from DiagXotics Co. Ltd. The gel on the left shows the control PCR product at 350 bp and the Thai product at 732 bp. The southern blot membrane on the right was prepared from the gel on the left and it shows that Thai HPV does not react so strongly with the DiagXotics labeled probe as does the DiagXotics control HPV product

Detection of HPV is not easy because there are no unique and distinctive gross signs associated with infection. Since it occurs only in the hepatopancreas (Fig. 20), the shrimp must be killed to be examined histologically. This is not problematic for larval samples, but it is for broodstock and pond reared shrimp. We need diagnostic reagents that can preferably be used with the shrimp feces, and DNA probes are ideal for such applications. Commercial DNA diagnostic probes and PCR primers for HPV are available (DiagXotics Co. Ltd., Wheaton, Conn.) based on an HPV isolate in *P. chinenis* (HPV-chin) from Korea, but it turned out that these were not ideal for HPV from Thailand. HPV in *P. monodon* (HPV-mon) from Thailand is quite different (Sukhumsirichart *et al.*, 1999) with a larger genome of 6 kb single stranded DNA compared to 4 kb of HPV-chin. The DiagXotics primer designed from HPV-chin DNA gives a 732 bp fragment with HPV-mon rather than the predicted one of 350 bp. When we compared the 732 bp fragment from HPV-mon to that of HPV-chin at GenBank, we discovered that HPV-mon had only 70% homology to HPV-chin (Phromjai *et al.*, 2001) and this explained the weak DNA hybridization in Southern blots (Figs. 21). To improve sensitivity with HPV-mon, the primers of Sukhumsirichart *et al.*, (1999) may be used instead of those of DiagXotics to yield an HPV specific fragment of 156 bp. This fragment is shown below with the primer sequences underlined.

```
5' -  GCA CTT ATC ACT GTC TCT ACC CAA GTC ATG AGC TGT CTG
3' -  CGT GAA TAG TGA CAG AGA TGG GTT CAG TAC TCG ACA GAC

      AAA GCC TTG TAT ATA TGG CAA CCA GAC TTT GCT CAA GAA
      TTT CGG AAC ATA TAT ACC GTT GGT CTG AAA CGA GTT CTT

      ATC CTC CTT CAT GGT TAG CAT TTT CAC AGC TAT ACT AAT
      TAG GAG GAA GTA CCA ATC GTA AAA GTG TCG ATA TGA TTA

      CTT ATG ACA GAG CAA GGT ATT TAC AAA GTT CAC -3'
      GAA TAC TGT CTC GTT CCA TA A ATG TTT CAA GTG -5'
```

One drawback of these primers is that they yield a very small PCR fragment that may be confused with primer-dimer pairs. As a result, we have designed a second set of primers for PCR amplification of a 441 bp specific DNA fragment from HPV-mon (submitted to DAO). These primers have also been used to produce a 441 bp dioxygenin labeled specific probe for HPV by PCR using the 732 bp cloned fragment as a template. These methods are currently being applied in Thailand for detection of HPV DNA in extracts derived from PL and faeces (Fig. 22) by PCR (Fig. 23) and dot blot hybridization (Fig. 24) and they are available commercially from the Shrimp Biotechnology Business Unit, National Center for Genetic Engineering and Biotechnology, Bangkok. The primer sequences are:

```
primer HPV441F: 5'  ACA CTC AGC CTC TAC CTT GT 3'
primer HPV441R: 5'  GCA TTA CAA GAG CCA AGC AG 3'
```

and the PCR protocol is: 95°C for 5 min followed by 40 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min with final extension at 72°C for 7 min. The expected amplicon is 441 bp. Other prime sequences and *in situ* probes have been described as well (Pantoja and Lightner, 2000, 2001).

One major mystery that remains unsolved is the source of HPV in the farming system. We have never seen it in hatchery larvae (unpublished) and there are, as yet, no published reports of it there. Nor did we find it in captive broodstock (Flegel *et al*., 1997b) although the sample was small and we used histology only since we did not have PCR techniques at the time. The first place we have seen it with certainty is in nursery tanks to which the postlarvae are transferred from the hatchery for outdoor acclimatization before stocking in shrimp ponds. This suggests that there may be existence of an unknown reservoir carrier(s) of the virus. DNA probes would be useful in identifying this carrier(s) as a prelude to excluding it from the cultivation system.

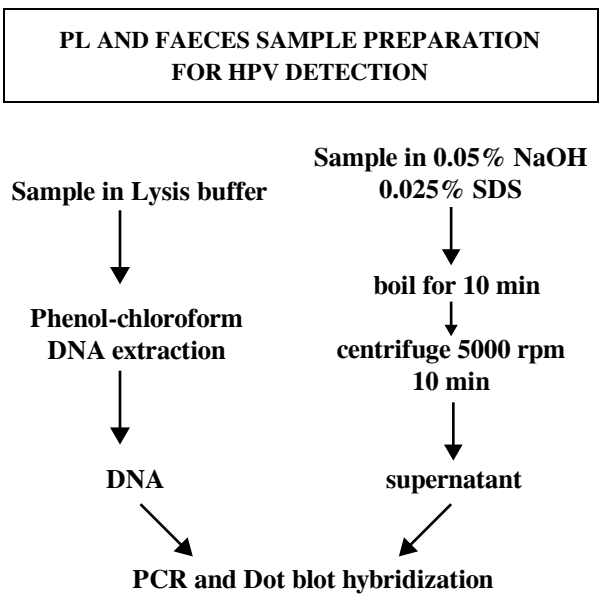


Figure 22. Procedures for preparation of DNA extracts from PL and faeces for PCR detection and dot blot detection of HPV and other DNA viruses. The lysis solution on the right can be used with fresh material only but has the advantage that no DNA extraction step is necessary. Normal lysis buffer (50 mM Tris-HCl pH 9, 100 mM EDTA, 50 mM NaCl, 2% SDS and Proteinase K 1 µg/ml added immediately before use) has the advantage that samples can be stored at room temperature for several years without degradation, but DNA extraction is necessary

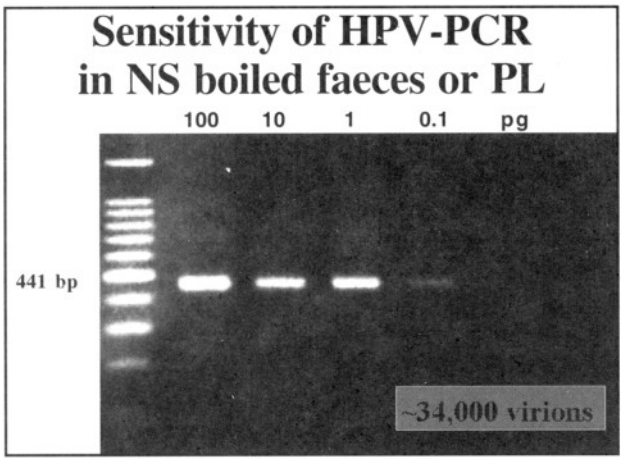


Figure 23. Example of PCR detection results for boiled PL or faeces spiked with HPV DNA at various concentrations. Due to extraction losses and possible inhibitory substances, the sensitivity for was limited to approximately 34,000 virion equivalents per gram of original fresh weight sample

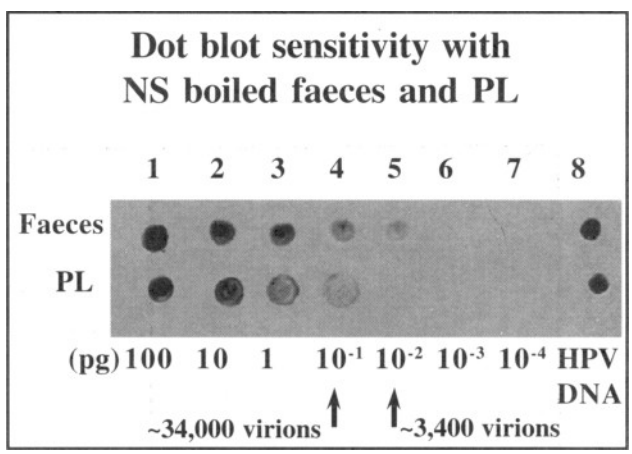


Figure 24. Example of a dot blot assay for HPV using fresh faeces or PL samples boiled in sodium hydroxide/ SDS solution

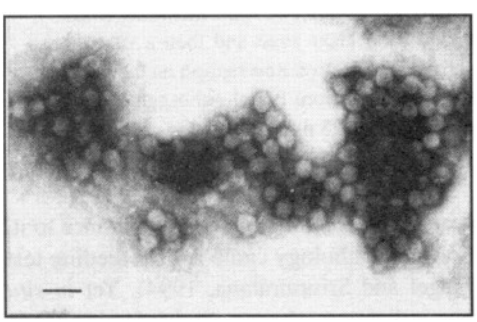


Figure 24a. Negatively stained transmission electron micrograph of purified virus particles from HPV-mon. Bar = 50nm. Like other typical parvoviruses, the virions are unenveloped, icosahedral and very small (around 24 nm in diameter)

INFECTIOUS HYPODERMAL AND HAEMATOPOEITIC NECROSIS VIRUS (IHHNV)

There is little hard data available for this virus in Thailand (Flegel *et al*., 1995a). However there is some circumstantial evidence for its presence. First of all, IHHNV is probably endemic in Asian *P. monodon* (Lightner, 1996; Lightner and Redman, 1991). Secondly, we have found histopathology typical of the virus on one occasion in apparently healthy shrimp (Flegel *et al*., 1992b) (Fig. 25) and the relevant specimens were confirmed for IHHNV by *in situ* hybridization (D.V. Lightner, unpublished). Third, we have frequently encountered paracrystalline arrays of 23 nm virus-like particles, which might be those of IHHNV, during examination of lymphoid organ specimens by electron microscopy (Flegel *et al*., 1997b) (Fig 25). Fourth, Thai shrimp farmers have complained about the increasing tendency for wide size variation in harvested crops, a feature of IHHNV infection in *P. vannamei* in which IHHNV infection causes runt deformity syndrome rather than massive mortality (Lightner, 1993, 1996). We still do not know whether IHHNV has any impact on the Thai shrimp culture industry. The situation requires further study, and commercial DNA diagnostic probes developed for IHHNV in Dr. Lightner's laboratory are available for rapid detection by *in situ* hybridization or by PCR assay using shrimp haemolymph (DiagXotics, Wilton CT). We have tried these reagents and they do work with Thai material (Fig. 26), so there are no technical obstacles to the work.

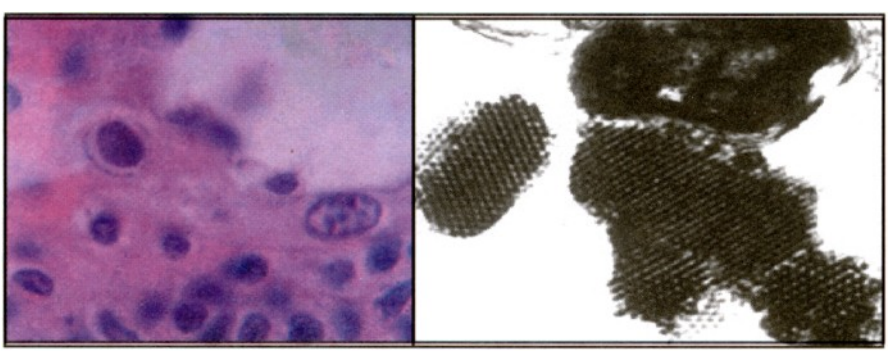


Figure 25. IHHNV from Thai shrimp. The photomicrograph on the left shows one nucleus of the antennal gland with a typical IHHNV Cowdry-A type inclusion (red, acidophilic, central inclusion surrounded by a clear zone and then a ring of marginated purple, basophilic, chromatin). The electron micrograph on the right shows what may be IHHNV viral particle arrays from lymphoid organ tissue. The viral particles in the array are approximately 23 nm in diameter

One curious feature of this virus is what appears to be *P. monodon's* high tolerance to it. The Thai specimens in which we found typical IHHNV histopathology came from a feeding test group that exhibited normal growth and survival (Flegel and Sriurairatana, 1994). Yet *in situ* hybridization tests gave very strong positive reactions, indicating a heavy viral infection (D.V. Lightner, pers. com.). Unfortunately, no bioassay could be performed using *P. stylirostris* with these specimens. There are also anecdotal reports of IHHNV tolerant or "resistant" *P. stylirostris* from Tahiti which were found to transmit virulent IHHNV to naive *P. stylirostris* in bioassay trials

(D.V. Lightner, pers. com.). This scenario seems to have common features with the accommodation to WSSV and YHV in Thailand, and in the case of IHHNV, it is clear that the shrimp have changed, not the virus.

Primers for the PCR detection of IHHNV appear in the OIE Diagnostic Manual for Aquatic Animal Diseases (2000). These give a 356 bp IHHNV specific fragment. The sequence of the primers is as follows:

Sense:	5' -	ATC	GGT	GCA	CTA	CTC	GGA	3'
Antisense:	5'	TCG	TAC	TGG	CTG	TTC	ATC	3'

It is possible that PCR labeling of the 356 bp fragment would yield a probe suitable for either dot blot DNA hybridization or *in situ* hybridization, but this would have to be ascertained by appropriate testing.

The full sequence of IHHNV is now available at GenBank and we have done a comparison of our HPV-mon sequence and the full sequence of HPV-chin at GenBank with that of IHHNV. Although HPV and IHHNV are both parvoviruses, it was interesting to discover that there was no significant homology between the sequences of the HPV strains and that of IHHNV. We have also done comparative dot blots with HPV and IHHNV probes and targets and we have found no cross-hybridization.

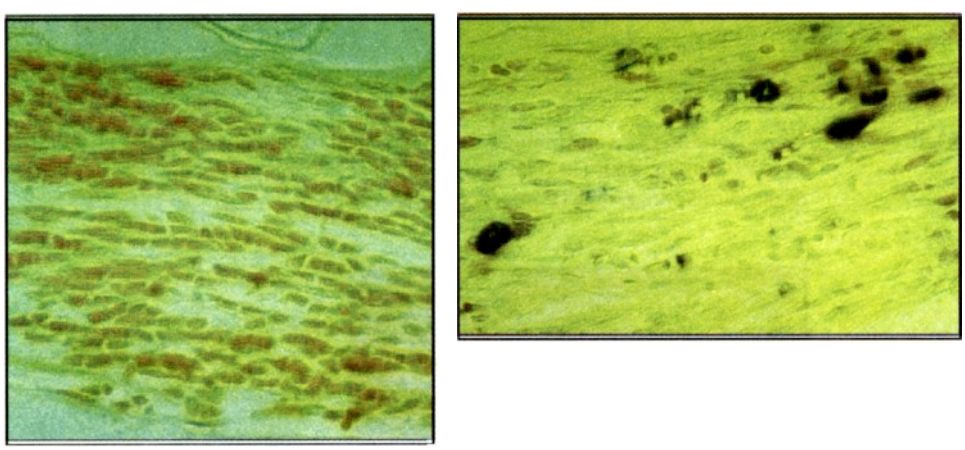


Figure 26. Example of a positive *in situ* hybridization reaction of DiagXotics commercial probe for IHHNV with shrimp from Thailand. The photomicrograph on the left shows a negative reaction with normal uninfected nerve tissue, while that on the right shows a positive reaction (blackened areas)

TAURA SYNDROME VIRUS (TSV)

The newest viral pathogen to arrive on the Asian scene is Taura syndrome virus (TSV) (Lightner, 1996; Tu *et al*., 1999). Taura syndrome was first recognized as a new disease in the Americas in 1992 but its viral etiology was not established until 1994 (Brock *et al*., 1995; Brock *et al*., 1997; Hasson *et al*., 1995). The causative agent has been tentatively classified as a member of the Picorniviridae because it is an unenveloped. 32 nm icosahedral virus containing a 10.2 kb ssRNA genome of positive sense (Bonami *et al*., 1997). TSV infections present characteristic gross pathology in *P. vannamei* that can serve from presumptive diagnosis (Fig. 27). These include reddening of the tail fan and visible necrosis of the epithelial tissue there in the acute phase of the disease. In the recovery or chronic phase of survivors, black lesions in the cuticle are often found. However, histological examination is required for confirmation of the disease (Fig 28). By TEM icosahedral virions can be seen in the cytoplasm of infected cells (Fig. 29).

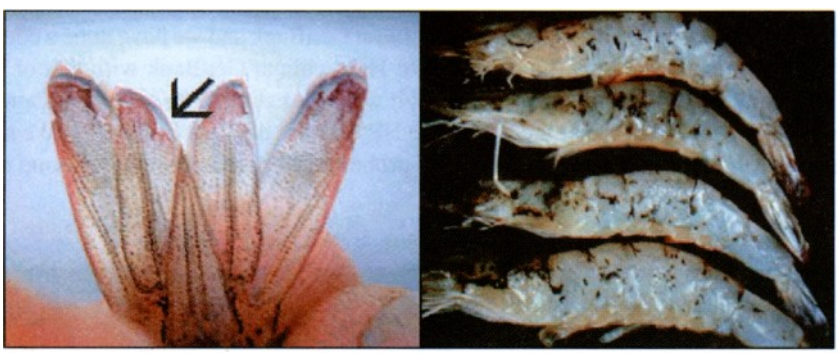


Figure 27. Gross signs of Taura syndrome. On the left is a tail fan of *P. vannamei* with reddish necrotic areas (arrow). The right photo shows black lesions in the cuticle characteristic of the chronic stage of TSV infection (Lightner, 1996)

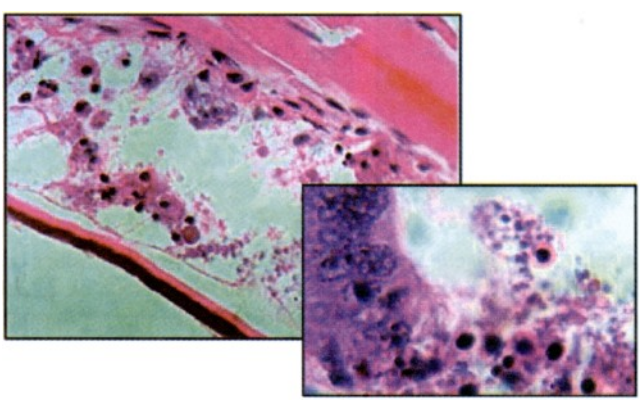


Figure 28. Histopathology of TSV infected *P. vannamei* in the acute phase of infection. Note the large masses of spherical, cytoplasmic inclusions that begin as eosinophilic to light basophilic bodies and later become intensely basophilic

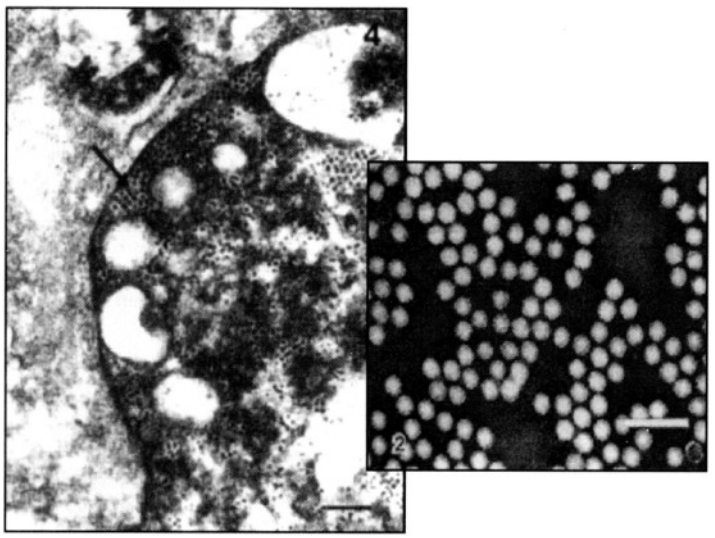


Figure 29. TSV by TEM. The electron micrograph on the left shows TSV in the cytoplasm by normal TEM using thin tissue sections from TSV infected *P. vannamei.* The micrograph on the right shows purified TSV virions by negative staining. (From Nunan *et al*., 1998).

TSV outbreaks were first reported in Asia from Taiwan where *P. vannamei* had been imported live as fry and brooders for use in commercial aquaculture ponds (Tu *et al*., 1999). It was probably introduced with the imported stocks but molecular epidemiological tests would be needed to confirm this. Although TSV is not highly lethal to *P. monodon*, its effect on other species of Asian shrimp is not known.

Molecular DNA methods for the detection of TSV have been reported (Mari *et al*., 1998; Nunan *et al*., 1998). Primers for an RT-PCR method that yields a 231 bp TSV specific fragment (Fig. 30) have been reported (Nunan *et al*., 1998). The primers are:

Sense:	5' TCA-ATG-AGA-GCT-TGG-TCC	3'
Antisense:	5' AAG-TAG-ACA-GCC-GCG-CTT	3'

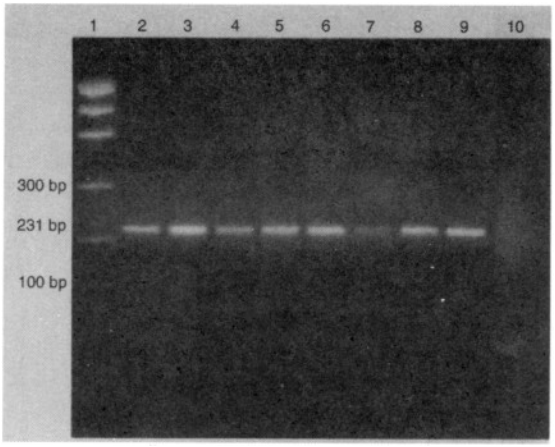


Figure 30. Agarose gel of PCR products from shrimp infected with TSV. Lane 1, 100 bp ladder; lanes 2-8, samples from shrimp injected with TSV and infection confirmed by histology; lane 9 positive TSV control; lane 10 negative control sample from pre-injection shrimp (Nunan *et al*., 1998)

VIBRIO PARAHAEMOLYTICUS AND *V. PENAECIDA*

Species of *Vibrio* are often the cause of shrimp death, although we believed that they are usually opportunistic pathogens that overcome shrimp defenses when they are weakened by some sort of predisposing stress (Flegel *et al.* 1995a). The way to solve the problem is to remove the cause of the underlying stress. The main *Vibrio* species responsible for shrimp mortality are *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, and perhaps *V. panaeicida*. We first worked on *V. parahaemolyticus* because it is also a serious human pathogen and a target for screening of frozen shrimp for export from Thailand. A probe was developed by random cloning and selection procedures and it was subsequently utilized for a rapid and sensitive PCR detection that can be used directly with shrimp haemolymph samples (Rojlorsakul *et al*., 1998) (Fig. 31). This system can be used to non-destructively check shrimp haemolymph samples without the necessity of traditional steps for bacterial isolation, purification and nutritional testing to obtain an identification. Like the other PCR assays described, the process takes only a few hours and contrasts sharply with the traditional methods which require several days and may risk misidentification of atypical strains. It is hoped that a cheap multiplex system will eventually be developed which would allow for the simultaneous PCR assay for all of the major *Vibrio* pathogens, but this will probably take a few years to reach fruition.

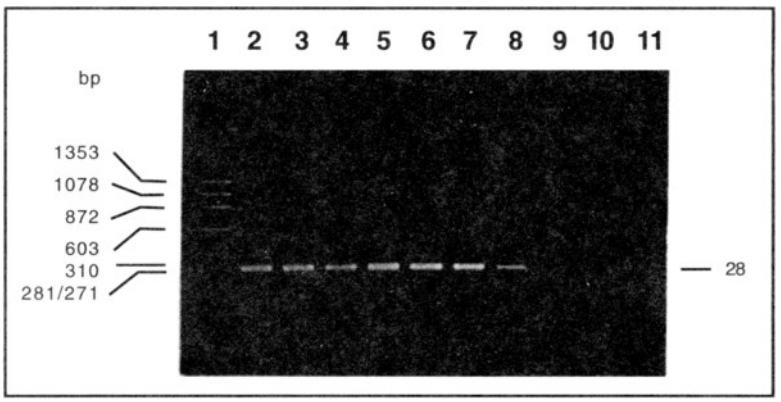


Figure 31. Agarose gel of PCR products from shrimp haemolymph samples containing cells of *V. parahaemolyticus.* The highest sensitivity seen in the gel is for lane 8 using a sample containing 2 x 10^4 bacterial cells per ml of haemolymph and 100 cells per PCR reaction vial

The PCR primers that yield the 285 bp fragment are as follows:

Sense:	5' -GTT ACG CAC AGA TGC GAC AT-3'
Antisense:	5' -CTT GTG GAT TGG ATT CTC GC-3'

Another species of bacterium that appears to cause high mortality in *P. monodon* is *V. penaeicida* (Ishimaru *et al*., 1995). This bacterium does not grow well on TCBS agar and may sometimes be overlooked in examining shrimp for bacterial infections. An RT-PCR method has been published for *V. penaeicida* from Japan (Genmoto *et al*., 1996) and a PCR method has been published for *V. penaeicida* from New Caledonia (Saulnier *et al*., 2000).

THE MICROSPORIDIAN *AGMASOMA PENAEI*

This intracellular parasite infects both *P. monodon* and *P. merguiensis*, but apparently does not usually cause very high mortality (Flegel *et al*., 1992a). It is most damaging because it disfigures the shrimp with white discoloration of the musculature ("white-back" or "cotton" shrimp) and reduces the selling price considerably. The parasite seems to be a bigger problem with *P. merguiensis* and it is probably the major reason that it cannot be used as an alternate species to *P. monodon*, even when higher prices for it or its resistance to yellow-head virus infection, for example, would make its cultivation advantageous.

To address this problem, we have tried to find the source of the pathogen in the shrimp cultivation system. When direct infection tests between shrimp failed, we began to look for an intermediate host. To do this, DNA diagnostic reagents were developed and used to screen potential reservoir (*i.e*., alternate) hosts (Pasharawipas and Flegel, 1994; Pasharawipas *et al*., 1994; Pasharawipas *et al*., 1997). By this process, two fish species (*Scatophagus argus* and *Priacanthus*

tayenus) were identified as potential hosts (Fig. 32). However, bioassay tests with one of these (*Scatophagus argus*) and shrimp have not yet been successful in closing the life cycle of the parasite, so the issue of the alternate host is still open.

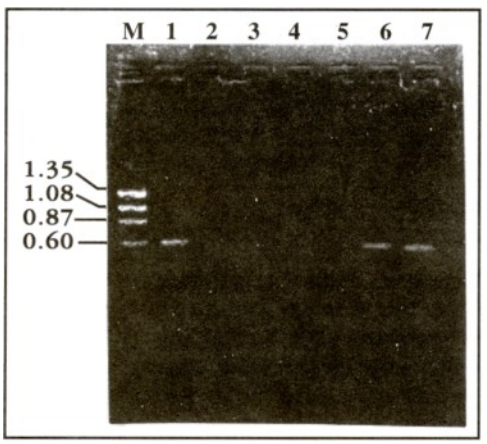
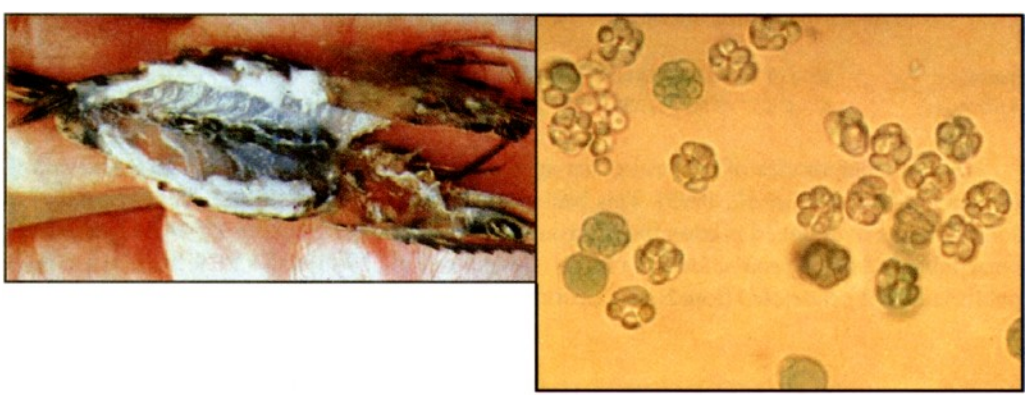


Figure 32. Agarose gel of PCR products using *Agmasoma*-specific primers with DNA extracts from *Scatophagus argus* and *Priacanthus tayenus* as the template. Lane M = molecular marker; lane 1 = 600 bp product from *Agmasoma* positive control template; lanes 2-5, no product from various negative controls including bacterial, protozoan and normal shrimp DNA templates; lanes 6 and 7, positive PCR product from the respective fish DNA templates



Additional figure. The photograph on the left shows the gross appearance of white shrimp tissue infected with the microsporidian *Agmasoma penaei.* The photomicrograph on the right shows a fresh mount from infected tissue with immature sporoblasts stained with malachite green while mature sporoblasts with 8 spores each are refractory to staining

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Diagnostic and Preventive Practices for WSSV in Japan

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ABSTRACT

White spot syndrome (WSS), considered equivalent to PAV (penaeid acute viremia) in Japan, has become the most serious problem not only in the farming industry but also in hatcheries for sea ranching of kuruma prawn, *Penaeus japonicus.* The prevalence of WSSV (white spot syndrome virus), the causative agent of WSS, was examined in wild kuruma prawn broodstocks by nested PCR (polymerase chain reaction). As a result, WSSV was detected at the highest prevalence (10.1%) in the ovary of female prawn. This result indicates that spawners are sources of infection. In 1997, brooders were pre-screened using PCR to detect WSSV before these spawned. WSSV was noted to occur in postlarvae obtained from brooders caught between July and August. In 1998 and 1999, eggs were selected based on WSSV detection by PCR from *receptaculum seminis* of spawned broodstock. Consequently, WSSV did not occur in their offsprings in both years. These results strongly indicate that selection of eggs based on PCR results is a practical way of controlling WSSV in hatcheries.

INTRODUCTION

In 1993, a viral disease caused serious mortalities in the shrimp farming industry of western Japan (Nakano *et al*., 1994). This viral disease, affecting the kuruma prawn *Penaeus japonicus*, was called penaeid acute viremia (PAV), and the causative virus was named penaeid rod-shaped DNA virus (PRDV) (Inouye *et al*., 1996). It is considered that PAV and PRDV are equivalent to WSS (white spot syndrome) and WSSV (white spot syndrome virus), respectively (Lo *et al*., 1996; Takahashi *et al*., 1996). In Japan, WSSV has damaged the farming industry of other shrimp species, as well as the shrimp hatcheries used in sea ranching (Momoyama *et al*., 1997; Satoh *et al*., 1999). In Southeast Asian countries, WSSV was reported in kuruma prawn, black tiger shrimp *P. monodon,* redtail shrimp *P. penicillatus,* and Chinese prawn *P. chinensis* (Chou *et al*., 1995; Peng *et al*., 1995; Wongteerasupaya *et al*., 1995; Lightner, 1996). The present study focused on the epizootiology of this viral disease in both seed production and nursery culture farms from 1996 to 1999. It was found that elimination of eggs from WSSV-positive spawners by polymerase chain reaction (PCR)-based technique was an effective control measure against WSSV in seed production.

MATERIALS AND METHODS

Broodstock and collection of eggs

Adult kuruma prawn, caught in coastal waters of Kyushu and Shikoku islands in Japan, were purchased from dealers and used as spawners between May and September 1996, and between April and August 1997. Transport time from collection areas to the Station of the Japan Sea-Farming Association (JASFA) was 4-10 h.

In 1996, 300-600 spawners were transferred in a 1.6-ton tank filled with sand-filtered seawater. In 1997, 1-7 spawners were placed in a 10 L container filled with UV (30,000 $\mu W/m^3$) - sterilized water. The water temperature in the tank was kept at 20-25°C in 1996 and 15°C in 1997, to suppress breeding of the spawners. Upon arrival at the Station, the spawners were immediately placed in a 35-ton spawning tank and maintained at 23-27°C for 1-3 days to induce spawning. In 1997, the ovaries (0.1 g) of all spawners were sampled using a disposable syringe (3.0 ml; needle, 19G), and WSSV was detected individually by PCR. Only spawners with WSSV-negative ovaries (5-12 individuals) were placed in the spawning tank at 24-28°C to induce spawning.

In 1998 and 1999, 5-18 non-biopsied individuals were stocked separately in 0.5 m^3 spawning tanks at 23-25°C to induce spawning. The *receptaculum seminis* was sampled for WSSV detection by PCR from the spawned broodstocks. Based on the PCR results of the *receptaculum seminis*, only the eggs from WSSV-negative spawners were used in the rearing experiments.

The fertilized eggs in the 1996 spawning were washed with clean filtered seawater, while the eggs during the 1997-1999 spawnings were disinfected with 5mg/l povidone-iodine for 5 min. The eggs were temporarily stocked in 200 L rearing tanks until the end of the process of WSSV detection by PCR. Only the eggs from WSSV-negative spawners were reared in rearing tanks (150-2,500 ton). The water temperature was kept at 24-28°C.

Seed production

Filtered and UV-sterilized seawater was used for seed production in 1996-1999. The hatched nauplii were fed with *Tetraselmis tetrathele*, nauplii of *Artemia salina* and commercial formulated feed. From the hatchery, the shrimp were transferred to nursery facilities and stocked in concrete rearing tanks where water temperature was maintained between 25-28°C. For WSSV detection by PCR, samples were collected at egg, nauplius, zoea, mysis, and postlarval (PL) stages (egg: 0.05 mg, mean body weight; PL1: 1 mg; PL5: 1.5 mg; PL10: 3 mg; PL20: 12 mg; PL30: 30mg; and PL40: 100 mg). In each sampling stage up to PL10, the total amount of the sample was 0.1 g. From PL20-PL40, 30 PL were collected at each sampling time. In nursery facilities, samples were collected at 10-day interval.

Detection of WSSV from wild adult broodstock

Broodstock were captured in five different areas (central Honshu, Shikoku, and Kyushu) of the coastal waters of Japan from July 1996 to April 1998. Samples of hemolymph, stomach, and gonad of wild female (955 individuals) and male (314 individuals) prawn were submitted to WSSV detection by PCR as described below. The mean body weight of females and males were 78.1-105.5 and 44.7-63.9 g, respectively. The hemolymph was collected using a syringe (1 ml; needle, 26G), and 500 μl was mixed with phosphate buffer saline (PBS, pH 8). The stomach and gonad samples were aseptically extracted at a volume of 100 μg, and stocked at -80°C until the PCR analysis.

DNA extraction and detection of WSSV by PCR

The hemolymph, stomach, gonad, *receptaculum seminis*, and whole body of juveniles were individually homogenized and digested with ISOGEN (Japan Gene Co.). The total DNA was amplified using two specific primer sets, P1/P2 and P3/P4 (Kimura *et al*., 1996). After 30 cycles amplification for each primer set at 93°C (60 sec), 57°C (90 sec), and 72°C (60 sec), the amplified products were analyzed by agarose gel electorophoresis. A known WSSV-infected *P. japonicus* was processed as a positive control.

RESULTS

Occurrence of WSSV in seed production

In 1996, WSSV was detected in 4 out of 9 seed production runs, thus the larvae were discarded (Table 1). The developmental stage in which WSSV was first detected by PCR was in eggs, followed by PL5 and PL10, when the water temperature was 22.1-29.4°C. In the other seed production runs, WSSV was detected in PL29 and PL51 at the nursery stage. High mortality rates reaching 50-100% occurred within 10 days in the nursery facilities after detection of WSSV. Moribund juveniles showed red coloration and discoloration of the body, and white spots on the carapace. In 1997 when selection of spawners was based on WSSV detection from ovaries before spawning, WSSV was not detected in a total of 9 seed production trials and 17 nursery cultures (Table 2).

Table 1. Detection and occurrence of white spot syndrome virus (WSSV) in *Penaeus japonicus* in 1996 at Japan Sea Farming Association (JASFA)

Trial No.	Spawners		Detection of WSSV in seed production (eggs to PL20)*2	Occurrence of WSSV	
	Captured date	PCR test*1		In seed production	In nursery culture
1	May 24, 25	-	-	-	- (0/4)*3
2	June 25	-	-	-	- (0/1)
3	July 12	-	-*4 (PL51)*4	-	+ (3/3)
4	July 16, 17	-	-	-	- (0/3)
5	July 24	+	-*4 (PL29)*4	-	+ (5/5)
6	Sept. 3	+	+ (Eggs)	+ (Discarded)	Not done
7	Sept. 4	+	+ (Eggs)	+ (Discarded)	Not done
8	Sept. 12	+	+ (PL10)	+ (Discarded)	Not done
9	Sept. 17	-	+ (PL5)	+ (Discarded)	Not done

*1 Detection of WSSV in the cuticular epidermis of stomach of spawners after spawning by 2-step PCR

*2 Detection of WSSV in larvae and postlarvae sampled during seed production. The stage of prawn in parenthesis represents the developmental stage when WSSV was first detected by 2-step PCR

*3 Number of WSSV cases recorded/conducted

*4 WSSV was detected later in juveniles which were reared in the hatchery after their siblings were transferred to nursery facilities

Table 2. Detection and occurrence of white spot syndrome virus (WSSV) in *Penaeus japonicus* in 1997 at Japan Sea Farming Association (JASFA)

Trial	Spawners	Detection of WSSV from postlarvae*1	Occurrence of WSSV	
	Captured date	PCR test	In seed production	In nursery culture
1	April 11	-	-	- (0/1)*2
2	May 12, 13	-	-	- (0/1)
3	May 16	-	-	- (0/4)
4	May 17	-	-	- (0/1)
5	May 12, 13	-	-	- (0/2)
6	June 11, 12	-	-	- (0/3)
7	July 17	-	-	- (0/3)
8	July 19	-	-	- (0/1)
9	July 22	-	-	- (0/1)

*1 Detection of WSSV in postlarvae just before transport to nursery culture facility
*2 Number of WSSV cases recorded/conducted

Detection of WSSV *from wild adult prawns*

The prevalence rate of WSSV showed high values: [ovary (10.1 %)]>[stomach (7.3 %)]>[hemolymph (5.8 %)] in females as shown in Fig. 1. In males, WSSV was detected in the stomach (6.7 %) and spermatheca (4.8 %), but not in the hemolymph (Fig. 1).

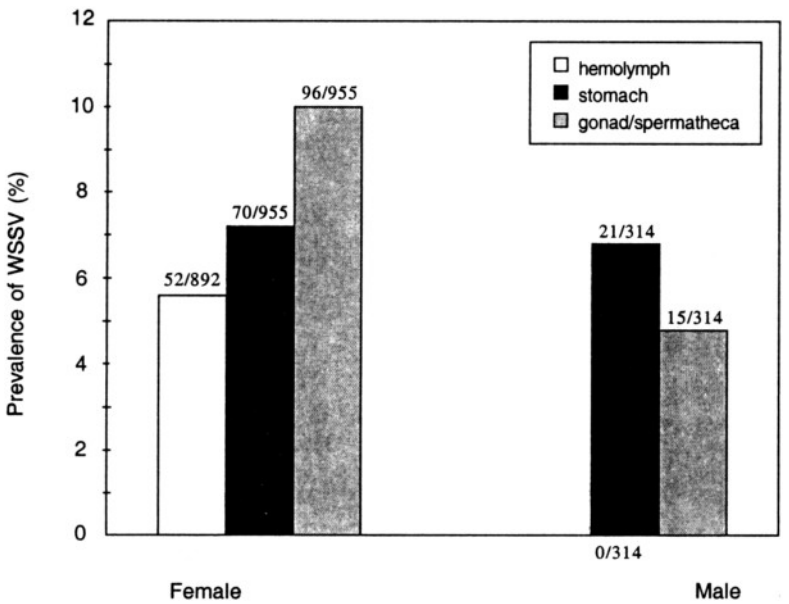

Figure 1. Prevalence of white spot syndrome virus (WSSV) detected by polymarase chain reaction (PCR) in wild adult Kuruma prawn (*Penaeus japonicus*) captured at 5 different coastal waters from 1996 to 1998

Detection of WSSV before and after spawning

The results of WSSV detection in the ovary and *receptaculum seminis* of spawners before and after spawning are shown in Table 3. The prevalence of WSSV in the ovary was 0.9% in 1997, and 0% in 1998 and 1999 before spawning. The values for ovary after spawning were 4.7% in 1997, 0.5% in 1998, and 1.9% in 1999. In the *receptaculum seminis*, the prevalence of WSSV before spawning was 5.6% in 1997, 0% in 1998, and 2.1% in 1999, whereas after spawning the values were 33.5, 6.3 and 10.9%, respectively. Thus, WSSV was detected in the *receptaculum seminis* at a higher rate after spawning than before spawning, and its prevalence increased rapidly from June onwards.

Table 3. Prevalence of WSSV in ovary and *receptaculum seminis* of *Penaeus japonicus* spawners before and after spawning in 1997-1998 at Japan Seafarming Association (JASFA)

Date of purchase		Prevalence of WSSV (%)			
		Ovary		*Receptaculum seminis*	
		Pre-spawning	Post-spawning	Pre-spawning	Post-spawning
1997	April	1.6 (4/248)*1	3.4 (3/87)	NE*2	NE
	May	0 (0/81)	0 (0/40)	NE	NE
	June	0 (0/108)	0 (0/ 37)	0 (0/38)	2.3 (2/86)
	July	0.7 (2/297)	4.0 (4/101)	8.6 (6/70)	39.2 (83/212)
	August	1.3 (3/240)	10.0 (10/100)	5.7 (2/35)	52.6 (41/78)
Total in 1997		0.9 (9/974)	4.7 (17/365)	5.6 (8/143)	33.5 (126/376)
1998	March	0 (0/3)	0 (0/18)	0 (0/3)	0 (0/18)
	April	0 (0/38)	0 (0/587)	0 (0/38)	1.4 (8/587)
	May	0 (0/13)	0.9 (1/111)	0 (0/13)	0.9 (1/111)
	June	0 (0/13)	1.6 (2/122)	0 (0/13)	13.9 (17/122)
	July	0 (0/108)	1.4 (2/148)	0 (0/21)	24.3 (36/148)
Total in 1998		0 (0/175)	0.5 (5/986)	0 (0/88)	6.3 (62/986)
1999	March	0 (0/15)	0 (0/181)	0 (0/15)	0 (0/181)
	April	0 (0/10)	0 (0/262)	0 (0/10)	0 (0/262)
	May	0 (0/5)	0 (0/39)	0 (0/5)	0 (0/39)
	June	0 (0/10)	6.8 (5/74)	0 (0/10)	41.9 (31/74)
	July	0 (0/7)	0 (0/15)	14.3 (1/7)	56.1 (37/66)
Total in 1999		0 (0/47)	1.9 (12/622)	2.1 (1/47)	10.9 (68/622)

*1 PCR positive/examined
*2 Not examined

DISCUSSION

In the 1996 production run, WSSV did not occur until PL20. However, WSSV was detected after the shrimps were transferred to the nursery farms. In 1997 after spawner selection and disinfection of eggs with iodine as WSSV control measures, WSSV was not detected in both seed production and nursery phases of culture. From these results, the major infection route of WSSV in seed production was considered to be a vertical transmission (Satoh *et al*., 1999).

From the results of WSSV detection by PCR in wild adult kuruma prawn, the stomach is not a suitable target organ for WSSV detection. But the ovary or *receptaculum seminis* of spawners is. It was also shown in the present study that the use of eggs from WSSV-negative spawners was an effective way of controlling WSSV in seed production. Moreover, WSSV was detected in the *receptaculum seminis* at a higher rate after spawning than in ovaries before spawning (Mushiake *et al*., 1999). Therefore, the use of *receptaculum seminis* is recommended as the target organ for WSSV detection for broodstock selection. Since 1998 when these procedures were adopted, WSSV has not occurred in the seed production of kuruma prawn at JASFA for the past 3 years (Table 4). These measures are effective.

Table 4. Occurrence of white spot syndrome virus (WSSV) in *Penaeus japonicus* in 1996 to 1999 at Japan Sea Farming Association (JASFA)

Year	Selection of spawners*1	Disinfection treatment of eggs	No. of success/ total conducted
1996	Not done	Not done	6/14
1997	From ovary (before spawning)	Iodine	21/23
1998	From R. S.*2 (after spawning)	Iodine	11/11
1999	From R. S. (after spawning)	Iodine	16/16

*1 Selection based on PCR results
*2 R.S. : *receptaculum seminis*

The increase in the WSSV detection rates from wild-captured kuruma prawn in summer (July to August) corresponds with the result of *P. monodon* (Lo *et al*., 1996). This was probably caused by stress due to multiple spawnings between March and September. The possibility that multiple spawnings could induce viral multiplication in the host has been shown in viral nervous necrosis of the striped jack *Pseudocaranx dentex* (Mushiake *et al*., 1994). There is no data identifying a similar phenomenon in kuruma prawn, however, the possibility might exist because the spawning season of kuruma prawn ranges between April and October and this species repeats copulation and spawning several times in one season.

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Diagnostic and Preventive Practices for Iridovirus in Marine Fish

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ABSTRACT

The first outbreak of red sea bream iridoviral disease (RSIVD) caused by red sea bream iridovirus (RSIV) was recorded among cultured red sea bream (*Pagrus major*) in 1990 in Ehime, Shikoku, Japan. Since then, the disease has caused mass mortalities of many cultured marine fishes. From 1990-2000, RSIVD was detected in 31 cultured marine fish species, including 28 Perciformes, 2 Pleuronectiformes and 1 Teteraodontiformes, in 18 prefectures in the southwestern part of Japan. The infected fish are lethargic and show severe anemia, petechiae of the gills, and enlargement of the spleen. Histopathologically, the disease is characterized by the presence of enlarged cells in the spleen, heart, kidney, liver and gills that are deeply stained with Giemsa solution.

Diagnostic methods for RSIV, such as the observation of stained imprints or tissue sections, an immunofluorescent (IF) test with a monoclonal antibody (MAb) and a polymerase chain reaction (PCR) technique have been developed. The IF test with MAb is commonly used in the rapid diagnosis of RSIV-infected fish. For an effective control measure against RSIVD, a formalin-killed vaccine has been developed and this showed a significant effect in red sea bream under both experimental and field conditions.

INTRODUCTION

Iridoviruses, which are recognized as causative agents of serious systemic diseases, have been identified from more than 20 fish species in recent years (Hyatt *et al*., 2000). The family Iridoviridae comprises of large isometric viruses with icosahedral symmetry, 130-300 nm in diameter, with a genome of double stranded DNA, replicating only in the cytoplasm.

Red sea bream iridoviral disease (RSIVD) is one of the newly emerging major diseases in the aquaculture industry. The first outbreak of RSIVD caused by red sea bream iridovirus (RSIV) was recorded among cultured red sea bream, *Pagrus major*, around Shikoku Island, Japan in 1990. Since 1991, the disease has caused mass mortalities of many species of cultured marine fish. This paper briefly reviews RSIVD and recent studies on its diagnosis and vaccination trials against the disease.

RED SEA BREAM IRIDOVIRAL DISEASE

The affected fish are lethargic and exhibited severe anemia, petechiae of the gills and enlargement of the spleen (Inouye *et al*., 1992). The disease is characterized by the appearance of enlarged cells which stained deeply with Giemsa solution in tissue sections of the spleen, heart, kidney, liver, and gills of infected fish (Inouye *et al*., 1992). The most typical histological change observed in affected fish is the appearance of enlarged cells in the spleen.

The causative agent is a large, icosahedral, cytoplasmic DNA virus classified as a member of the family Iridoviridae (Inouye *et al*., 1992). The virus was first isolated in red sea bream and thus was designated as RSIV. Each virion consists of a central electron-dense core (120 nm) and an electron-translucent zone, measuring 200-240 nm in diameter. The biological and physico-chemical properties of the virus have been reported (Nakajima and Sorimachi, 1994). The RSIV can grow on GF, BF-2, CHSE-214, FHM, JSKG, KRE-3, RTG-2 and YTF cell lines. The titers of the virus are higher on GF, BF-2 and KRE-3 than on other cell lines. The virus can replicate at 15, 20, 25 and 30°C but not at 37°C. The optimum temperature for viral growth is 20°C or 25°C. The virus is sensitive to acid (pH 3), chloroform, ether, and heat but is not sensitive to ultrasonic treatment and repeated freezing and thawing. Treatment with 5-iodo-2-deoxyuridine (IUdR) reduced the titer of the virus.

The virions of RSIV contain linear double-stranded DNA. The complete nucleotide sequence of RSIV has been determined. The genome of RSIV is about 112,000 base pairs (bp) in length and contains about 90 potential genes (Kurita, unpublished data).

The antigenic relationship between RSIV and two iridovirus-like agents associated with systemic infection in fish, the epizootic haematopoietic necrosis virus (EHNV) and iridovirus isolated from sheatfish (SFIV), has been examined. Although cross-reactions were observed between RSIV and other fish iridoviruses by immunofluorescence (IF) or immunoprecipitation test using anti-RSIV serum, none of the monoclonal antibodies (MAbs) against RSIV reacted with EHNV- or SFIV-infected cells by the IF test (Nakajima *et al*., 1998). Pathogenicity tests of EHNV or SFIV to red sea bream have not been shown by experimental challenge (Nakajima and Maeno, 1998).

The RSIVD has affected 31 species of cultured marine fish in 18 prefectures in the southwestern part of Japan from 1990-2000 (Matsuoka *et al*., 1996; Kawakami and Nakajima, 2002). The infected fish include species belonging to Perciformes, Pleuronectiformes and Tetradontiformes (Kawakami and Nakajima, 2002). The affected fish are yellowtail, sea bass, Japanese parrotfish, amberjack (*Seriola dumerili*), goldstriped amberjack (S. *aureovittata*), striped jack (*Pseudocaranx dentex*), horse mackerel (*Trachurus japonicus*), albacore (*Thunnus thynnus*), Japanese flounder (*Paralichthys olivaceus*), and tiger puffer (*Takifugu rubripes*).

Transmission of the disease to healthy red sea bream has also been established by co-habitation or through the rearing water of RSIV-infected fish (Nakajima, unpublished data). These suggest a possibility of horizontal transmission of RSIVD. Until now, RSIVD has not occurred in hatcheries; thus, the possibility of vertical transmission seems to be little.

DIAGNOSIS OF RSIVD

Diagnostic methods such as the observation of stamped or sectioned specimens stained with Giemsa, an IF test with a MAb and a polymerase chain reaction (PCR)-based technique have been reported (Inouye *et al*., 1992; Nakajima and Sorimachi, 1995; Nakajima *et al*., 1995; Oshima *et al*., 1996, 1998; Kurita *et al*., 1998). The diagnosis of RSIVD by an IF test with a MAb is done either by isolation of RSIV in cell culture followed by its identification with an anti-RSIV M10 MAb or direct demonstration of antigens in infected tissue using M10 MAb. The indirect IF test using an M10 MAb revealed that specific fluorescence was observed in tissue imprints or frozen sections of spleen of red sea bream and other fish (Nakajima *et al*., 1995). Thus, the indirect IF test with a MAb is commonly used for the rapid diagnosis of RSIV-infected fish in the field.

The localization of the reactive antigen with M10 MAb has been examined by immuno-electron microscopy. The reactive antigen was not found in the virion with M10 MAb, but an antigen was detected on the surface of the virus-infected cells (Nakajima, unpublished data). These results suggest that the MAb recognizes a virus-induced polypeptide located on the surface of the virus-infected cells.

A PCR technique was developed to detect RSIV using primers based on the sequence data of RSIV. Four oligonucleotide primer sets based on the ATPase gene, DNA polymerase gene and a Pst I-restriction fragment of RSIV genomic DNA were synthesized to amplify the RSIV DNA of 563-570 bp length (Kurita *et al*., 1998). Furthermore, since the target region among RSIV was successfully amplified from diseased fish other than red sea bream, the PCR using primers designed for RSIV has a broad application for the diagnosis of RSIVD in a number of species (Kurita *et al*., 1998).

VACCINATION AGAINST RSIVD

The effectiveness of vaccination against RSIVD has been evaluated using two kinds of vaccines in red sea bream under experimental conditions (Nakajima *et al*., 1997). For vaccine preparation, RSIV-infected GF cells or its cell culture supernatant were inactivated with formalin (1.0% v/v or 0.3% v/v) for 10 days at 4°C. Juvenile red sea bream were intraperitoneally injected with the vaccine, and after 10 days the fish were RSIV-challenged by intraperitoneal injection. Statistical analysis showed significantly higher survival rates in the vaccinated groups than that of the respective control groups, indicating the protection was induced by vaccination.

In addition, the expression of the virus specific antigens in the spleen was monitored by IF test for both the vaccinated and control fish after RSIV challenge. The results show that the expression of antigens was weaker in the vaccinated fish compared with the control fish supported the efficacy of the vaccination.

To establish control measures for RSIVD, the effectiveness of a formalin-killed viral vaccine was evaluated in a field trial (Nakajima *et al*., 1999). Two groups each consisting of 1000 juvenile red sea bream were either intraperitoneally inoculated with the vaccine (vaccinated group) or were not vaccinated (control group). After vaccination, the fish were held in tanks for one week, then transferred to a marine net pen and observed for 12 weeks. The cumulative mortalities

caused by RSIVD in the vaccinated group and control group were 19.2 and 68.5%, respectively. Additionally, the presence of virus antigen in the spleen was investigated and body weight was measured 6 and 12 week post-vaccination. In the vaccinated group, viral antigen was not detected. In contrast, viral antigen was detected in the control group at 6 week post-vaccination, but was not detected at 12 week post-vaccination. The increase in body weight of vaccinated fish was significantly ($p<0.05$) greater than that of control fish. These results suggest that the vaccine against RSIVD was effective in the field trial.

The vaccine was also found to be effective in other cultured marine fish such as the yellowtail (*S. quinqueradiata*). The vaccine for red sea bream and yellowtail is now commercially available and is currently used for vaccination against RSIVD in Japan.

DISCUSSION

The outbreak of RSIVD was first reported in 1990. Although the original source of infection of the disease is not clear, importation of the seedling fish from foreign countries without any quarantine is suspected to have introduced the new pathogen into Japan. In relation to infectious source, comparison between RSIV and other iridovirus isolated in foreign countries especially in Southeast Asia where Japan has imported a lot of seedlings is needed.

Control measures against infectious diseases of cultured fish include the following: avoidance of exposure to the pathogen, environmental manipulation, immunization, activation of non-specific defense, development of disease resistant-strains, health maintenance, and chemotherapy. Among them, activation of non-specific defense and specific immunization based on general health maintenance seem to be the most promising prophylactic methods for the control of RSIVD. Breeding of red sea bream for disease resistant-strains has been left for further studies.

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Diagnostic and Preventive Practices for Viral Nervous Necrosis (VNN)

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ABSTRACT

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) caused by piscine nodaviruses (=betanodaviruses) has spread worldwide in the past decade among cultured marine fish. The present paper briefly describes procedures currently practiced in the diagnosis and prevention of the disease.

INTRODUCTION

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) caused by piscine nodaviruses has spread worldwide, *i.e.* in Indo-Pacific region, Mediterranean, Great Britain, Scandinavia, and North America, among cultured marine fish with the number of susceptible host species continuing to grow (Munday and Nakai 1997; Curtis *et al*., 2001; Starkey *et al*., 2000; Grotmol *et al*., 1997). To date, the disease has been reported in at least 25 fish species, with the greatest impact being in sea bass *Lates calcarifer* (Glazebrook *et al*., 1990) and *Dicentrarchus labrax* (Breuil *et al*., 1991), groupers *Epinephelus akaara* (Mori *et al*., 1991), *E. fuscogutatus* (Chi *et al*., 1997), *E. malabaricus* (Danayadol *et al*., *1995), E. moara* (Nakai *et al*., 1994), *E. septemfasciatus* (Fukuda *et al*., 1996), *E. tauvina* (Chua *et al*., 1995), *E. coioides* (Lin *et al*., 2001) and *Cromileptes altivelis* (Zafran *et al*., 2000), striped jack *Pseudocaranx dentex* (Mori *et al*., 1992), parrotfish *Oplegnathus fasciatus* (Yoshikoshi and Inoue, 1990), puffer *Takifugu rubripes* (Nakai *et al*., 1994), and flatfish *Verasper moseri* (Watanabe *et al*., 1999), *Hippoglossus hippoglossus* (Grotmol *et al*., 1997; Starkey *et al*., 2000), *Paralichthys olivaceus* (Nguyen *et al*., 1994), and *Scophthalmus maximus* (Bloch *et al*., 1991).

The causative agent was first characterized in striped jack *P. dentex* and then in barramundi *L. calcarifer,* European sea bass *D. labrax,* and grouper *Epinephelus* sp. with VNN. (Mori *et al*., 1992; Comps *et al*., 1994; Chi *et al*., 2001). It is currently placed in the genus Betanodavirus, the family Nodaviridae (van Regenmortel *et al*., 2000). Immunological studies have shown relationships between striped jack nervous necrosis virus (SJNNV, the type species of the genus Betanodavirus) and the other betanodaviruses (Munday *et al*., 1994; Totland *et al*., 1999; Skliris *et al*., 2001). The genomic classification of betanodaviruses, based on partial nucleotide sequences of the coat protein gene (RMA2), has also shown that major genotypes are the SJNNV-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type and red spotted grouper nervous necrosis virus (RGNNV)-type (Nishizawa *et al*.,

1997). The complete nucleotide sequences of RNA1 and RNA2 (RNA-dependent RNA polymerase) of SJNNV or GGNNV (a grouper betanodavirus) have been reported (Iwamoto *et al,* 2001; Tan *et al*., 2001).

In the past decade, information on the disease and betanodaviruses has accumulated and there are now many procedures available for diagnosis of the disease. The present paper briefly describes the diagnostic methods of VNN and the procedures to prevent the disease, although the prevention method has not been fully developed because of unknown infection mechanisms of the disease, particularly in grouper's VNN.

DIAGNOSTIC METHODS

1. Clinical signs and histopathology

There are considerable variations in age at which the disease is first noted and the period over which mortality occurs. In general, the earlier the signs of disease occur, the greater is the rate of mortality. Although disease occurrence at the juvenile stage in some species is very rare, high mortalities often occur at juvenile to young stages in other fish species, but mortality usually does not reach 100%, indicating age-dependent susceptibility (OIE, 2000). Mortalities have been reported in production-sized European sea bass (Le Breton *et al*., 1997) and sevenband grouper *E. septemfasciatus* (Fukuda *et al*., 1996).

All diseases are characterized by a variety of neurological abnormalities such as erratic swimming behavior and vacuolation of the central nervous tissues (brain, spinal cord) and the retina. In general, younger fish have more severe lesions; older fish have less extensive lesions and these may show a predilection for the retina (Munday and Nakai, 1997). Endocarditis has been described in Atlantic halibut (Grotmol *et al*., 1997) and neuronal necrosis has been described in most species.

Presumptive diagnosis of VNN can be made on the basis of a conspicuous vacuolation in the brain, spinal cord and/or retina. However, VNN in fish with only a few vacuoles in the nervous tissues maybe difficult to diagnose.

2. Electron microscopy

The virus particles can be visualized in affected brain, spinal cord, and retina. The virus is mainly associated with vacuolated cells and, characteristically, some inclusions. The reported particles vary in size from 22 nm (Breuil *et al*., 1991) to 34 nm (Yoshikoshi and Inoue, 1990) arranged intracytoplasmically in crystalline arrays, or as aggregates and single particles intra-and extracellularly. The virus is nonenveloped and icosahedral in shape.

3. *Immunological methods*

All betanodaviruses can be detected either by indirect fluorescent antibody test (FAT) or immunohistochemistry (IHC) with a rabbit anti-SJNNV serum (Munday and Nakai, 1997; Munday *et al*., 1994; Totland *et al*., 1999). These are routinely used for confirmative diagnosis of the disease. Although other immunological methods, e.g. the enzyme-linked immunosorbent assay (ELISA) (Arimoto *et al*., 1992; Huang *et al*., 2001) or neutralization test (Skliris *et al*., 2001), are available for virus identification, they may be used only for some betanodaviruses due to limited information on the serological properties of the virus.

There have been reports on the sero-diagnosis of VNN or VER using ELISA methods (Mushiake *et al*., 1992; Mushiake *et al*., 1993; Breuil and Romestand, 1999; Breuil *et al*., 2000). However, due to insufficient knowledge on the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not gained acceptance as a routine screening method for assessing the viral status of fish populations.

4. *Molecular methods*

Reverse transcription-polymerase chain reaction (RT-PCR) is the most powerful tool to detect the virus not only from diseased fish but also from asymptomatic carriers. A single primer set designed to amplify the T4 region (427 bases) of SJNNV coat protein gene (Nishizawa *et al*., 1994) is available for all genotypic variants of betanodaviruses, with only one exception (Thiery *et al*., 1999). Although RT-PCR is convenient for the diagnosis of VNN or VER, this technique requires $10^{4\text{-}5}$ TCID50 for detection of the betanodaviruses. The sensitivity of the diagnosis is improved by nested RT-PCR (Thiery *et al*., 1999; Della Valle *et al*., 2000). Comps *et al.* (1996) reported *in situ* hybridization using DIG-labelled probes to examine the tissue-specific expression of betanodaviruses.

5. *Culture in cells*

All genotypic variants of betanodaviruses can be cultured in the fish cell line SSN-1 which was derived from striped snakehead *Channa striatus* (Frerichs *et al*., 1991, Frerichs *et al*., 1996; Iwamoto *et al*., 1999), and a clonal cell line E-11 from the SSN-1 cells is useful for qualitative and quantitative analyses of all the betanodaviruses (Iwamoto *et al*., 2000). It is notable that both SSN-1 and E-11 cells are infected by a spontaneously productive C-type retrovirus designated as SnRV (Frerichs *et al*., 1991; Hart *et al*., 1996; Iwamoto *et al*., 2000). Virus titration and growth experiments using the E-11 cell line clearly revealed differences in the optimal growth temperature among the genotypic variants: 25 to 30°C for RGNNV genotype, 20 to 25°C for SJNNV genotype, 20°C for TPNNV, and 15 to 20°C for BFNNV. This culture system is also useful for detection of virus-neutralizing antibodies in fish serum (Tanaka *et al*., 2001; Yuasa *et al*., 2002). Needless to say, the culture system has opened a gate for molecular analysis of betanodaviruses (Iwamoto *et al*., 2001).

The E-11 cells are highly sensitive to betanodaviruses but it takes a maximum of 10 days after inoculation to detect the virus at lower numbers based on the CPE expression. In contrast, the RT-PCR is generally a rapid method to examine a large number of samples but its sensitivity is not so high, as mentioned above. Accordingly, the combined procedure of cell-culture and RT-PCR techniques will be a rapid and convenient method to detect infective viral particles from asymptomatic carriers or samples with low virus levels. For example, when the 72-h culture of virus in the E-11 cells was examined by the RT-PCR, a positive-PCR amplicon was obtained from every genetic variant sample containing virus particles at the lowest number (10° TCID50). The cultivation for 24 to 48 h prior to RT-PCR was enough to detect the virus at the lowest titer (Iwamoto *et al*., 2001). The validity of this procedure, *i.e.* preculture in the E-11 cells and RT-PCR, has been demonstrated in the detection of a RGNNV- genotype betanodavirus from white seabass *Atractoscion nobilis* (Curtis *et al*., 2001).

PREVENTIVE METHODS

1. Prevention of vertical transmission

In VNN of striped jack, virus-carrying broodstock were shown to be the most important inoculum source of the virus to their larvae (Arimoto *et al*., 1992; Mushiake *et al*., 1994). This finding led to successful control of VNN in larval striped jack. Elimination or segregation of virus-carrying spawners, based on RT-PCR or cell-culture procedure just prior to spawning, is highly effective to prevent the disease, where disinfection of fertilized eggs with ozone is usually practiced for extra safety (Mushiake *et al*., 1994; Mori *et al*., 1998). However, the transmission mode of causative viruses in fish other than striped jack remains unclear, although betanodaviruses have been demonstrated in the gonad materials of broodstock of groupers and European sea bass (Nakai *et al*., 1994; Comps *et al*., 1996).

2. Prevention of horizontal transmission

The mode of transmission/introduction of the viruses, other than in gametes, has not been demonstrated, but the possibilities include influent water, juvenile fish held on the same site, and carriage on utensils, vehicles, etc. As betanodaviruses are quite resistant to environmental conditions (Frerichs *et al*., 2000), it is possible that they are readily translocated by commercial activities. Horizontal transmission and introduction of the viruses via contaminated rearing water and utensils will be possible.

Anderson *et al.* (1993) reported that a regime of non-recycling of water, chemical sterilization of influent seawater and decontaminating half of the tanks during each hatching cycle was successful in preventing VNN in an Australian barramundi hatchery. In the control of VNN in larval striped jack, Arimoto *et al.* (1996) recommended the following measures: (1) disinfection of eggs with iodine or ozone and utensils with chlorine; (2) rearing of each batch of larvae/juveniles in separate tanks supplied with seawater sterilized by UV or ozone; and (3) rigorous separation of larvae/juveniles from broodfish.

3. *Vaccination*

Virus-neutralizing antibodies have been found in the serum of sevenband grouper that survived intramuscular injection with the betanodavirus, indicating establishment of acquired immunity in survivors. As many grouper species are susceptible to the virus even at the grow-out stage, vaccination is an alternative to control the disease. Intramuscular injection of the *Escherichia coli*-expressed recombinant virus coat protein, which was constructed from RGNNV genotype strains, induced virus-neutralizing antibodies in groupers and protection against challenge by homologous or heterologous RGNNV virus (Tanaka *et al*., 2001; Yuasa *et al*., 2002). Since this antibody-reaction is genotype-specific, a multivalent vaccine will be required for overall protection from infection by the different genotypic variants responsible for causing VNN. A similar vaccine-efficacy experiment was performed on juvenile turbot *S. maximus,* resulting in significant protection against the virus challenge (Husgaro *et al*., 2001). The authors also showed that intraperitoneal injection of an oil-emulsified recombinant protein from SJNNV induced a specific humoral immune response in both turbot and Atlantic halibut *H. hippoglossus.* This suggests a potential for the control of VNN through maternal immunity of spawners that received the recombinant vaccine.

4. *Reducing stress factors*

In spite of the above-mentioned procedures, ultimate prophylaxis must be to reduce various stress factors happening under culture conditions. Anderson *et al.* (1993) found that reducing the stocking density of barramundi to 10 larvae/1 or lower in green ponds can reduce transmission and disease to a negligible level. In the infection experiment with redspotted grouper *E. akaara*, Tanaka *et al.* (1998) reported that rearing water temperature (16-28°C) influences development of VNN: higher mortality and earlier appearance of the disease signs are observed at higher water temperatures, suggesting that manipulation of water temperature will be efficacious in reducing the disease outbreaks. Mushiake *et al.* (1994) recommended that provisionally virus-free fish (striped jack) are not induced to spawn more than 10 times in a season, because the stress of multiple spawning activates residual, extraovarian virus. Recently, a very strange phenomenon was found in hatchery-produced juvenile kelp grouper *E. moara* (Banu and Nakai, unpublished data). When apparently healthy fish in a pond were examined by RT-PCR, nodavirus was detected from the brains at high frequency. In order to follow-up this phenomenon, virus-free kelp grouper were injected intramuscularly with a betanodavirus. As a result, the inoculated virus was isolated from the brains and eyes at very high titers, the maximum 10^9 TCID50/g, but no disease signs were noticed in fish. Probably, unknown stress factors involved in rearing process of fish induce this inapparent infection to apparent infection. Therefore, it is essential to identify such stress factors in rearing of fish.

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Diagnostic Practices for Marine Fish Viral Diseases in Thailand

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ABSTRACT

The Department of Fisheries, Thailand has three institutions that are capable of virus isolation using fish cell culture system: the Aquatic Animal Health Research Institute (AAHRI), the National Institute of Coastal Aquaculture (NICA) and the Marine Shrimp Research and Development Center (MSRDC). The AAHRI is located in Bangkok while the others are in Songkhla province, south of Bangkok. Fish cell culture system was initiated in AAHRI and NICA in 1992-1993. Both institutions spent 6-12 months to develop and practice cell culture. Since then, fish cell lines have been utilized for virus isolation. Various rhabdoviruses, iridoviruses and reoviruses were isolated from diseased freshwater fishes as well as iridoviruses from cultured frog. In addition, iridoviruses and nodavirus were also isolated from diseased marine finfish. The AAHRI maintains 8 fish cell lines and 2 reptile cell lines while NICA maintains 3 fish cell lines. The MSRDC has 5 marine finfish cell lines. In the three institutions, Leibovitz -15 is the general culture medium used in both tissue culture flask and tissue culture plate systems. This medium is capable of maintaining the pH in close and open culture systems without CO_2 incubation.

Diagnostic practices for marine viral diseases in Thailand include virus isolation, histology and polymerase chain reaction (PCR) amplification technique. As diagnosis in virology is costly, only suspected virus-infected specimens submitted to the Aquatic Animal Disease Clinics are examined for viruses. An active surveillance program of marine viral diseases, with support from the Government of Japan-Trust Fund, has begun this year. The diagnostic procedures for marine viral diseases in the three institutions are similar to the techniques described in the Office International des Epizooties (OIE) Diagnostic Manual and Blue Book.

INTRODUCTION

Knowledge of viral diseases of aquatic animals is very important in the aquaculture industry. Diagnosis of viral diseases is one of the most complicated and time consuming work in aquatic animal disease laboratories. Diagnosis of viral diseases in finfish is more advanced in cold-water fish aquaculture such as the salmonids than the warm-water fishes because of the availability of expertise and funding. However, there is limited information on viral diseases in most countries in Asia.

Viruses usually cause high mortality in fry or juvenile fish. Very low mortalities due to viral infection are reported in larger or adult fish. However, adult fish may readily become infected that will possibly cause high losses. For viral infections, pathogenesis is directly related to the condition of the fish immune system. In tropical regions, lowering of environmental temperature usually reduces the ability of fish to resist viral infection.

In Thailand, initial work on viral diseases in finfish started in early 1990s at the Aquatic Animal Health Research Institute (AAHRI) and the National Institute of Coastal Aquaculture (NICA) and in mid-1990s at the Marine Shrimp Research and Development Center (MSRDC). The AAHRI was developed from the Fish Disease Group of the National Inland Fisheries Institute (NIFI). During the 15 years within NIFI, facilities and expertise in fish health research improved with support from various international agencies including FAO, UNDP, USAID, CIDA and IDRC. In 1990, the Overseas Development Administration (ODA) of the United Kingdom, now the Department for International Development (DFID), recognized the potential of the Fish Disease Group and developed it as a center of expertise in fish disease research for the Southeast Asia region. Subsequently, the Department of Fisheries of Thailand, with support from the ODA through the South East Asia Aquatic Disease Control Project, established AAHRI, which moved into its present premises in 1992. The staff and facilities were upgraded, and its function was expanded to include research on diseases of all aquatic animals.

CELL CULTURE COLLECTION

The AAHRI maintains eight fish cell lines and two reptile cell lines while NICA maintains three fish cell lines (Table 1). The MSRDC has five marine finfish cell lines.

Table 1. Cell line collection of Department of Fisheries, Thailand

Institution	Cell line code	Cell line, full name
AAHRI	EPC	Epithelioma papulosum cyprini
	BB	Brown bullhead
	BF-2	Bull gill fry
	FHM	Fathead minnow
	SSN-1	Striped snakehead fish
	SGP	Snakeskin gourami caudal peduncle
	DFT	Discus fish tail
	HCT	Hybrid catfish tail
	SCE	Siamese crocodile embryo
	STE	Soft-shelled turtle embryo
NICA	EPC, SSN-1, FHM	
MSRDC	SK	Sea bass kidney
	GF	Grouper fin
	GMF	Gray mullet fin
	GGF	Giant grouper fry
	HGF	Humpback grouper fin

The culture medium used for fish and reptile cell lines is Leibovitz-15 supplemented with 10% serum and 2 mM L-glutamine. All cell lines are maintained in an incubator without CO_2 at 25-28°C.

AQUATIC ANIMAL HEALTH CLINIC

All three institutions operate an Aquatic Animal Health Clinic that provides diagnostic facility for fanners. The AAHRI and NICA issue health certificates for live aquatic animal shipments for export. The staff members often visit fish farms at the farmer's request to provide further advice on disease prevention and health management.

DIAGNOSTIC PRACTICES FOR FISH DISEASES IN THAILAND

Generally, occurrence of disease is related to a number of factors such as fish host condition, environment and pathogens. Diagnostic practices conducted at Aquatic Animal Health Clinic are as follows:

1. Obtaining information from fish farmers: all the necessary information on aquaculture activities in the farm are recorded and evaluated.

2. Measurement of water quality parameters: basic parameters measured are pH, alkalinity, hardness, color and transparency.

3. Pathogen examination: diseased specimens are examined for pathogen according to their symptoms as different techniques apply to different pathogens. The details of general diagnostic procedures for finfish diseases in Thailand are documented in Tonguthai, et al. (1999).

Diagnostic Practices for Marine Fish Viral Diseases in Thailand

1. Tissue and fluid sampling

The selection of tissues for viral assays varies according to the size and maturity of the specimen. The following tissues are sampled (Ganzhorn and LaPatra, 1994):

Size/maturity of fish	Tissue assayed
< 4 cm	Entire fish (exclude yolk sac)
4-6 cm	Entire viscera (include kidney)
> 6 cm	Kidney, spleen and gills
Sexually mature	Ovarian fluid, kidney, spleen and gills

For large fish, portions of the kidney, spleen, pancreas and gills are combined as one sample. The tissue sample should be at least 1 g. The retina layer of the eye ball and the brain are taken for fishes exhibiting clinical signs of VNN and combined as one sample.

Tissues from a maximum of 10 fry/fingerlings are pooled and treated as one sample. When larger fish are sampled, tissues from less than five fish are pooled. Pooled samples are of equal volume or weight.

2. Preparation of Tissue Extract

Diseased fish with minor clinical lesions are collected. Fish are sacrificed and wiped clean with tissue paper. Approximately 1 g of fish tissue is needed. The tissue sample can be pooled from muscle and internal organs. For muscle samples, tissue debris and surface fungus on the ulcerated lesions are removed using a clean razor blade. Pieces of muscle tissue are taken from beneath the lesions. For internal organs, the abdomen is carefully opened using clean scissors and small pieces of tissue from kidney, spleen and pancreas are taken and pooled. These tissue samples can be stored up to 48 h in transport medium or Hank's balanced salt solution (HBSS) with 2% fetal calf serum, 500 units/ml of penicillin and 500 µg/ml of streptomycin. The samples can also be immediately processed as follows:

a. Samples are homogenized using a sterile, pre-cooled pestle and mortar until a smooth paste is obtained. Sterile fine sand is added to facilitate homogenization.

b. Samples are diluted 1:10 by the addition of 9 ml HBSS with 2% fetal calf serum. After mixing well, the samples are transferred to sterile centrifuge tubes and spun at 1000x g at 4°C for 15 min to separate cell debris, sand and possibly some contaminants from the fluid extract.

c. A further 1:5 dilution (or 1:50 from the original tissue sample) is carried out by filling 5 ml sterile disposable syringes with 4 ml HBSS with 2% fetal calf serum and then drawing up 1 ml supernatant.

d. The 1:50 final dilution is mixed well, then filter-sterilized through 0.45 µm disposable filter units.

e. The filtrates or tissue extracts are kept in 5 ml sterile bottle at 4°C and are then ready to be inoculated onto fish cell lines.

3. Viral Isolation

Simultaneous cell culture and sample inoculation are carried out using at least 2 different fish cell lines. For diseased marine fish, the extract is inoculated onto SSN-1 and EPC or SCE cell lines while SSN-1 or BF-2 and EPC are used for isolation of viruses from fresh water fish. Viral isolation is done in 24-well plates if there are many samples. The following are the general practices at AAHRI:

a. Each plate is first seeded with a single cell suspension of the fish cell line in maintenance medium (L-15 medium containing 2% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin).

b. Each well receives 1.3-1.4 ml of cell suspension. Cells with complete monolayer in 25 cm^2 tissue culture flask is sufficient to produce an 80-90% confluent monolayer in 1 day after seeding in one 24-well tissue culture plate.

c. One tissue extract (1:50 dilution) is immediately inoculated into two wells. The first well receives 200 µl inoculum while the second well receives 50 µl inoculum. The same number of replicate wells is used as negative control for each plate. However, a 1:10 dilution of the tissue extract is prepared if low viral infection is suspected.

d. The tissue extract-inoculated cells are incubated at 23-25°C and observed daily for CPE for at least 14 days. Observation time is longer for marine viruses.

e. A first blind passage of culture fluid is performed on days 7-10 for freshwater fish viruses. For marine fish viruses, the first blind passage is performed on days 10-14. Viral passage or subculture is done by transferring 200 µl of supernatant from each well to fresh 24-well culture plate. Observations for CPE is continued further for 5-7 days in the old plates. A second and third blind passages are also carried out.

f. Samples showing CPE in which the cell monolayer changed, disintegrated, sloughed off from the surface of the tissue-culture wells or ended with cell lysis, will be passaged further to provide larger quantities of the suspected virus.

g. A 500 µl of supernatant from a single well exhibiting CPE is inoculated into 25 cm^2 flasks containing 80-90% confluent cell monolayer. The suspected virus is allowed to be adsorbed for 1 h.

h. The cells are washed once with 5 ml PBS then 7-8 ml of maintenance medium is added.

i. Flasks are incubated at 23-25°C together with un-inoculated control flasks for comparison.

j. When the cells show complete CPE, they are centrifuged at 1000x g at 4°C for 15 min.

k. The supernatants are collected, aliquoted in tubes with 1 ml quantities and stored at 4°C and 20°C or -80°C, for further characterization.

VIRAL CHARACTERIZATION AND IDENTIFICATION

Once viruses are isolated in cell culture, efforts are needed to maintain them. Viruses are checked after storage to ensure viability. The basic properties of viruses, such as type of nucleic acid, envelop testing and particle morphology, are examined for viral Family classification.

Since the establishment of cell culture system in Thailand in the early 1990s, various fish rhabdoviruses, iridoviruses and reoviruses have been isolated from diseased freshwater fishes as well as iridoviruses from cultured frog. Iridoviruses and nodavirus have also been isolated from disease marine finfish.

For viral identification, serology and molecular biology are basically used. Polyclonal antibodies are produced from rabbits and neutralization and immuno-blot techniques are conducted for serological tests. A classic neutralization test is normally used to identify viruses and identify the sero-group. With the accessibility of different sequences of viral genes in database such as the Gene Bank, it is easy to obtain sequences that can be used for gene probes and primers for PCR.

With these advanced molecular tools, viruses are readily identified within a short time. An active surveillance program on viral diseases of grouper in Thailand using viral isolation and PCR screening has begun this year, with support from the Government of Japan-Trust Fund.

CONCLUSION

It does take time to get familiar with cell culture and learn how to prevent bacterial contamination, especially in laboratories where the culture medium is bought in powder form and pipettes and bottles are re-used. Most of the procedures for cell culture and virus isolation performed at the three institutes of the Department of Fisheries generally follow the standard procedures published elsewhere such as the OIE Diagnostic Manual (OIE, 2000) and the Blue Book (Thoesen, 1994)). Surprisingly, after the establishment of cell culture and virus isolation, many viruses have been isolated from diseased fishes in Thailand. Most of these viruses isolated were found to be new strains. It is most important to encourage scientists in the region to conduct researches in aquatic animal viruses, as viral diseases in many countries have not been explored.

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Progress and Current Status of Diagnostic Techniques for Marine Fish Viral Diseases at the SEAFDEC Aquaculture Department

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ABSTRACT

The incidence of unexplained mortalities among marine finfish in the Philippines has been increasingly observed. Considering that outbreaks of viral infections affecting similarly cultured marine fishes such as grouper and seabass were reported in many countries, a comprehensive diagnostic program to meet this challenge was initiated at the Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFDEC-AQD) with funding from the Japanese Trust Fund Fish Disease Project. This activity was further boosted by the Japan International Research Center for Agricultural Sciences (JIRCAS). Overall, the program involved the staff of the marine finfish hatchery and of the Fish Health Section. Cases of unexplained mortalities observed in the hatchery were referred to the Fish Health Section. Detailed information on the culture histories of each case were provided by the hatchery staff. Diagnostic tests were performed on each case and those with potential indication of viral etiology were processed for virus detection. Presumptive diagnosis of viral infections was based on typical signs, cell culture isolation, histopathology and *in-vivo* pathogenicity tests. Confirmatory tests to identify specific viruses include RT-PCR, FAT and electron microscopy. The highlights of outbreaks of viral nervous necrosis and other virus-associated infections among marine finfish at SEAFDEC-AQD are presented.

INTRODUCTION

Grouper (*Epinephelus* spp.), snapper (*Lutjanus argentimaculatus*), sea bass (*Lates calcarifer*), rabbitfish (*Siganus gutatus*) and milkfish (*Chanos chanos*) are high valued marine fishes with promising potentials in aquaculture. However, their successful culture had been hampered with outbreaks of viral infections (Yoshikoshi and Inoue, 1990; Mori *et al*., 1992; Munday *et al*., 1992; Anderson *et al*., 1993; Dayanadol *et al.* 1995; Chua *et al*., 1995; Boonyaratpalin *et al*., 1996; Fukuda *et al*., 1996; Le Breton *et al*., 1997; Chi *et al*., 1997; Chou *et al*., 1998; Curtis *et al*., 2001; Lin *et al*., 2001). In Asia, intensive research on these viral pathogens has been pursued in Japan, Taiwan, Singapore, China, Indonesia and Thailand. In the Philippines, investigations on

marine fish viral pathogens were revived recently through the support of the Fish Disease Project of the Japanese Trust Fund.

To date, there are three viral pathogens significant to the aquaculture of warmwater marine finfish namely: Viral nervous necrosis (VNN) virus, iridovirus and birnavirus. Hence, these are the viral pathogens that are the focus of the fish viral surveillance programme at SEAFDEC-AQD. The possible occurrence of new viral pathogens is, likewise, not overlooked. This report details the fish viral diagnostic procedures used and summarizes findings.

VIRAL DIAGNOSTIC SERVICES AT THE FISH HEALTH SECTION OF SEAFDEC-AQD

The diagnostic services of the Fish Health Section at SEAFDEC-AQD started in 1981. Initially, tests were limited to detection and identification of parasitic, bacterial and fungal etiologic agents. For fish viral etiology, diagnosis relied mainly on histopathology. In 1993, Lavilla detected typical vacuolations in the eyes of a diagnostic case of sea bass, *Lates calcarifer*, larvae at SEAFDEC-AQD (Lio-Po, 2001). It was subsequently confirmed as a classic case of VNN by B. Munday (Lavilla, pers. comm.). Eventually, cell cultures were established and maintained (Lio-Po *et al*., 1999; R. Fernandez, pers. comm..). As a result, the rhabdovirus associated with the epizootic ulcerative syndrome (EUS) was the first fish virus pathogen isolated in cell culture in the Philippines (Lio-Po *et al*., 2000). Due to budgetary and personnel constraints, virus isolation in cell cultures was not sustained.

In 2000, the Japanese Trust Fund Fish Disease Project was implemented at SEAFDEC-AQD. With this development and the availability of more cell lines and advanced molecular techniques for the detection of fish viral pathogens, research on fish virus was revived including fish virus detection in the diagnostic services of the Fish Health Section. Thus, the current Fish Health Section's diagnostic service for microbial infections is more comprehensive (Fig. 1). Fish samples from cases of unexplained mortalities were then processed for the isolation and detection of viral pathogens. In addition, parasitic, bacterial and fungal etiologies were ruled out for each case. The program involved the staff of the marine fish hatchery and of the Fish Health Section. Unexplained mortality cases and details on their culture histories were provided by the fish hatchery staff. Diagnostic tests were performed on each case and those with potential indication of viral etiology were processed for virus detection.

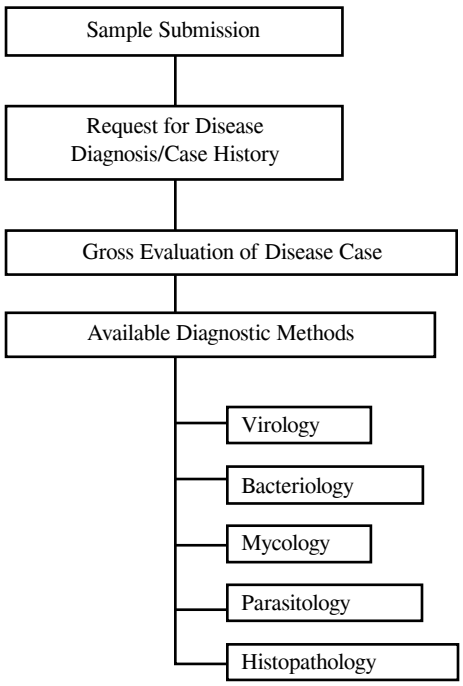

Figure 1. Flow chart of diagnostic sample processing

For viral diagnosis, samples were subjected to presumptive tests and then confirmatory tests to confirm viral etiology. Presumptive tests consisted of typical disease signs, cell culture isolation, histopathology and pathogenicity assay. The confirmatory diagnostic tests used were electron microscopy (EM), reverse transcriptase-polymerase chain reaction (RT-PCR) for detection of VNN and fluorescent antibody test (FAT) using monoclonal antibodies against iridovirus.

Cell Cultures

Established fish cell lines derived from fresh and marine fish species were maintained (Table 1). The cells were cultured in minimum essential medium (MEM) or Leibovitz medium (L15) with 10% fetal bovine serum (FBS). The pH of all media used was adjusted to 7.2 - 7.4 with 7.5% sodium bicarbonate and supplemented with 100 i.u. penicillin G sodium, 100 μg streptomycin sulfate, 25 μg amphotericin B per ml medium.

Table 1. Fish cell lines at SEAFDEC Aquaculture Department

Cell lines		Source
BF-2	(Bluegill fry)	N. Ohseko/Y. Maeno
CFS	(Catfish spleen)	G. Lio-Po/R. Hedrick
EPC	(*Epithelioma papulosum cyprini*)	K. Nakajima/N. Ohseko/Y. Maeno
FHM	(Fathead minnow)	K. Nakajima
GF	(Grouper fin)	M. Yoshimizu/J. Kasornchandra
SBK-2	(Sea bass kidney)	M. Yoshimizu
SHS	(Snakehead spleen)	G. Lio-Po/R. Hedrick
SSN-1	(Striped snakehead whole fry)	S. Kanchankhan/N. Ohseko/Y. Maeno
WSSk	(White sturgeon skin)	R. Hedrick
WSS2C1	(White sturgeon spleen clone 1)	R. Hedrick

Virus Isolation

External lesions and visceral organs (pool of spleen, liver, kidney, brain, eyes and gills) were aseptically excised and suspended in L15 medium containing 200 i.u. penicillin per ml and 200 μg streptomycin per ml. Tissue homogenates were prepared, diluted to 10% with Earle's balanced salt solution (EBSS) containing antibiotics and 50 μg amphotericin B per ml (antibiotic-antimycotic mixture A) (Lio-Po *et al*., 2000). The homogenates were then centrifuged at 3000 x g for 15 min at 4°C. The supernatant was filtered through a 0.45 μm pore size membrane filter (Millipore) and stored at -70°C until virus assay.

Primary viral isolations were conducted in EPC, SHS, FHM, SSN-1 cells in 24-well plates (Falcon) with MEM or L15 medium containing 2% FBS (L15-4) buffered with 1M N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid (Hepes). The plates were then incubated at 25°C for 7 days. Blind passages were carried out on negative samples at least three times. Only isolates manifesting consistent cytopathic effects (CPE) in all subsequent passages were considered positive. Viral titers were determined following the method applied by Lio-Po *et al*. (2000).

Pathogenicity Bioassay

In-vivo viral effects were determined by pathogenicity assays following the method of Lio-Po *et al*. (2001). Aliquots of either tissue filtrates or supernatants of cell culture assays positive for CPE were inoculated into healthy, susceptible fish by either intraperitoneal or intramuscular injection. The manifestation of disease signs in the test fish similar to that observed

in natural infection was interpreted as pathognomonic of viral infection. In addition, further viral assays of tissue filtrates derived from the experimental fish should show the presence of the inoculated virus in cell assays on susceptible cell lines.

Histopathology

For detection of typical histopathological lesions, sections of fish organs (kidney, brain, eyes, spleen) were fixed in 10% buffered formalin or Bouin's solution for at least 24 h then replaced with 70% ethanol. Fixed tissues were processed and sectioned following standard histological techniques (Luna, 1968). Histological sections were stained with Hematoxylin and Eosin stains then analyzed by light microscopy.

Electron Microscopy

In the absence of a transmission electron microscope at SEAFDEC-AQD, electron microscopic visualization of viral isolates was tentatively conducted in Japan in collaboration with JIRCAS scientists. Where appropriate, electron microscope facilities at the University of the Philippines or the St. Luke's Hospital in Manila, may also be tapped. Briefly, virus were inoculated onto susceptible cells in L15 medium and virus purified from cell-free supernatants by gradient ultracentrifugation. Negative staining were applied on purified virus. Infected cell cultures were fixed in glutaraldehyde and post-fixed with osmium tetroxide. Processed cells were embedded, sectioned and stained with uranyl acetate and lead citrate for transmission electron microscopy viewing.

RT-PCR for Detection of VNN

Two methods of tissue sampling were used: the destructive method and non-destructive method. Tissue samples collected for non-destructive sampling include eggs, milt, gills and blood. For destructive sampling, the whole fish was processed for larvae up to 15 days old. In larvae greater than 15 days old only the head portion was processed. For destructive sampling in adult fish, the brain and eyes were processed.

Tissue samples weighing 50-100 mg were dissected aseptically and homogenized in 1 ml of TRIzol reagent (Gibco) and centrifuged at 10,000 x g for 10 min. The supernatant was mixed with 2001 chloroform, centrifuged at 10,000 x g for 15 min. The aqueous phase was separated, added with 500 l isopropyl alcohol and incubated at room temperature for 10 min. After centrifugation at 10,000 x g for 10 min, the RNA was pelletted by adding 1 ml of 75% ethanol. RNA pellets were dried at room temperature, dissolved in DEPC-treated distilled water and incubated at 58C for 10 min. RNAs were stored at -80C until use.

Detection of the virus by RT-PCR amplification was carried out according to the procedure described by Nishizawa et al. (1994) selecting the T4 (430 base pairs) region as the target sequence for PCR amplification. Briefly, complementary DNA was synthesized from extracted RNAs using MMLV reverse transcriptase (Gibco) and reverse primer (R3:5'-CGA GTC AAC ACG GGT GAA GA-3') at 42C for 30 min and at 99C for 10 min. After addition of the forward primer (F2:5'-CGT

GTC AGT CAT GTG TCG CT-3') and Taq DNA polymerase (Gibco) to the mixture, each cycle of amplification was repeated 30 times at 95C (40 sec), 55C (40 sec) and 72C (40 sec) using a thermal cycler (Mastercycler gradient, Eppendorf). Amplified DNA was analyzed by agarose gel electrophoresis using 2% agarose (Agarose 1000, Gibco).

Fluorescent Antibody Technique (FAT) for Iridovirus Detection

This diagnostic technique was based on the method of Nakajima *et al.* (1995). The spleen of a fish was obtained, dissected and used to dab onto a clean slide. The impression smear was air-dried then fixed in pre-chilled acetone (-20C) for 10 min. After drying, the slide was stored at -20C until further use.

The fixed imprints of the spleen were covered with monoclonal antibodies (M10) and incubated at 37C for 30 min in a humid chamber. The slide was then rinsed three times for 1 min with phosphate buffered saline (PBS) by dipping 10 times in each beaker. The slide was overlain with the secondary antibody (anti-mouse IgG, Cappel) and incubated at 37C for 30 min in a humid chamber. After the slide was washed three times with PBS, two drops of glycerol was added as mounting fluid. The slide was examined under a fluorescent microscope to identify immunofluorescence positive cells.

CASES PROCESSED FOR VIRAL PATHOGENS IN YEARS 2000~2001

Under the Japanese Trust Fund Project, processing of fish diagnostic samples for fish viral pathogens were conducted following the above-mentioned procedures since October 2000 to date. In 2000, a total of 15 fish cases suspected of viral etiology were processed for cell culture inoculation in EPC, FHM, GF, SBK-2 and SNN-1 cells. Of these specimens, filtrates from 4 rabbitfish cases, 5 grouper fingerlings cases and one snapper case were positive for virus.

The following year, samples of 33 cases of grouper eggs, fry/larvae, juvenile and broodstock; 6 cases of milkfish eggs, larvae and juveniles; 1 case of seabass larvae; 3 cases of rabbitfish juveniles and 6 cases of snapper eggs and juveniles were assayed for virus in CFS, EPC, FHM, GF, SBK-2, SHS, SSN-1 and WSS2C1 cells. Only samples from grouper eggs and larvae were positive for virus.

The use of RT-PCR test for VNN at SEAFDEC-AQD were initiated in 2001. This test detected the presence of VNN in 20 cases (43%) of grouper and in a case of snapper larvae. Histopathologic analyses of grouper larvae and fingerlings likewise showed the presence of typical vacuolations in the brain samples of 8 cases (17%) (Fig. 2). Furthermore, Maeno *et al.* (2002) experimentally reproduced VNN in healthy fish and demonstrated the virus by TEM (Fig. 3), thereby confirming the occurrence of VNN in grouper, *E. coioides* in the Philippines.

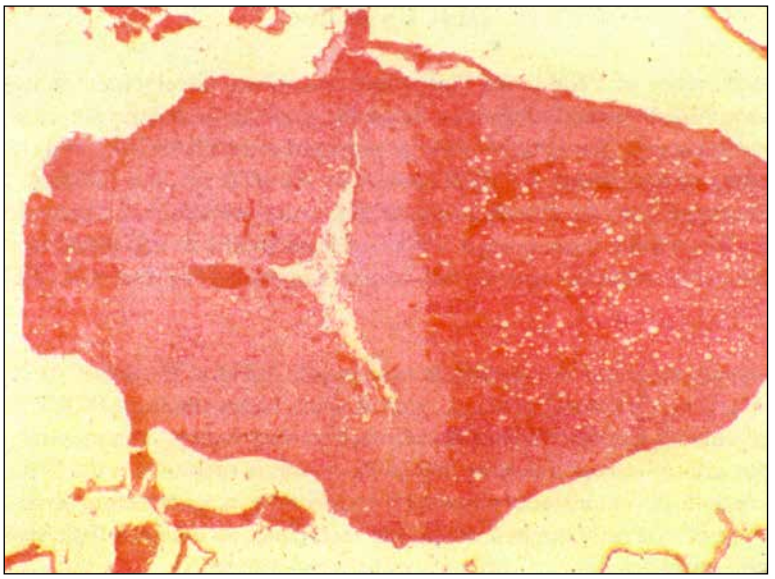

Figure 2. Histological section of the brain of grouper larva showing vacuolations (Hematoxylin and Eosin, 40x)

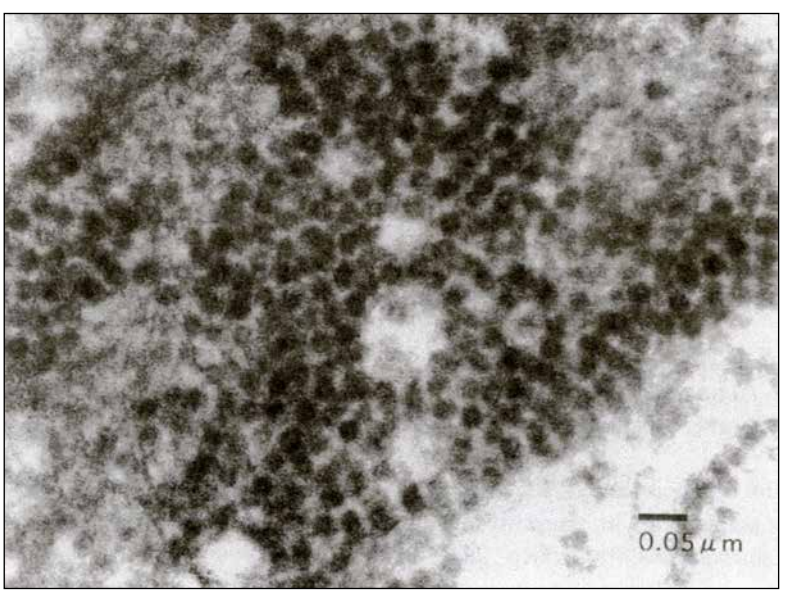

Figure 3. Transmission electron photomicrograph of the piscine nodavirus isolated in SSN-1 cells (bar= 0.05 μm)

DISCUSSION

The occurrence of VNN in the Philippines is a critical development in the successful culture of marine finfish in the Philippines. Since there are no treatments for viral infections, preventive measures are the best options and were proposed at the SEAFDEC-AQD fish hatchery. For one, screening of broodstock for VNN carriers by RT-PCR test was initiated. In addition, washing fertilized eggs with ozone-treated seawater following Arimoto *et al*.'s procedure (1996) is being planned. Vaccination trials using a VNNV vaccine similar to that tested in groupers, *E. septemfasciatus* and *Cromileptes altivelis* will be also be pursued (Tanaka *et al*., 2001; Yuasa *et al*., 2002).

In summary, diagnosis of viral infections in marine finfish at SEAFDEC-AQD was based on presumptive and confirmatory tests, *e.g*., typical signs, histopathology, PCR/RT-PCR, virus isolation in cell culture, pathogenicity tests and electron microscopy. The presence of VNN in grouper, snapper and sea bass as well as an unidentified virus in rabbitfish in the Philippines were confirmed. Meantime, development of more rapid diagnostic tests for detection of viral pathogens and improvement of currently applied diagnostic techniques to enhance their sensitivity and specificity for the early detection of viral pathogens is recommended.

ACKNOWLEDGMENTS

Appreciation is due to SEAFDEC-AQD and the Japanese Trust Fund for research support. We also thank Marietta Duray, Drs. Joebert Toledo, Gerald Quinitio, Arnil Emata and Felix Ayson for the fish samples and Michelle Peñaranda, Remia Traviña, Milagros Paner and the Microtechnique staff for technical assistance.

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HUSBANDRY METHODS FOR CONTROLLING LUMINESCENT VIBRIOSIS IN SHRIMP AND CRAB AQUACULTURE

Probiotics in Aquaculture

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ABSTRACT

The skin, lateral line, gills and gastrointestinal tract or a combination of these organs are suggested to be infection routes in fish. This presentation will present some information on pathogenesis, protection against bacterial adhesion, autochthonous microbiota in the gastrointestinal tract and prebiotics. This information is important when discussing the use of probiotics in aquaculture.

Intensive fish production has increased the risk of infectious diseases and there is a growing need to find alternatives to antibiotic treatment for disease control as indiscriminate use of antibiotics in many parts of the aquaculture industry has led to the development of antibiotic resistance in bacteria. Today, a range of microorganisms have been suggested as or evaluated as fish probiotics. These include lactic acid bacteria, *Bacillus* species, *Pseudomonas* species, *Vibrio* species and other Gram-negatives. However, research in probiotics for aquaculture is at an early stage and much work is still needed.

Another aspect on fish health is the use of prebiotics to increase the population level of already beneficial bacteria colonizing the gastrointestinal tract and the effect of diet in disease resistance.

INTRODUCTION

Before presenting the hard facts on probiotics in aquaculture a short background about pathogenesis, protection against bacterial adhesion and the autochthonous microbiota in the gastrointestinal tract is first provided here. This background information is important when discussing the use of probiotics in aquaculture. Pathogenic microorganisms have evolved mechanisms to target skin, gills or gastrointestinal tract as a points of entry, and today it is generally accepted that the three major routes of infection in fish are through: (a) skin (Kawai *et al*., 1981; Muroga and de la Cruz, 1987; Kanno *et al*., 1990; Magarinos *et al*., 1995; Svendsen and Bøgvald, 1997; Spanggard *et al*., 2001); (b) gills (Hjeltnes *et al*., 1987; Baudin Laurencin and Germon, 1987; Svendsen *et al*., 1999); and (c) gastrointestinal tract (Sakai, 1979; Rose *et al*., 1989; Chair *et al*., 1994; Olsson, 1995; Grisez *et al*., 1996; Olsson *et al*., 1996*;* Romalde *et al*., 1996; Jöborn *et al*., 1997; Robertson *et al*., 2000; Lødemel *et al*., 2001).

Pathogenicity can be divided into four different phases (Birkbeck and Ringø, 2002): (1) the initial phase where the pathogen enters the host's environment, including the gastrointestinal tract; (2) the exponential phase where the pathogen adheres to and colonizes mucosal surfaces, replicates to sufficient numbers and translocate into host enterocytes; (3) the stationary phase where the pathogen replicates within the host and circumvents the host defence system. In this phase the host is moribund and this can quickly be followed by (4) the death phase.

In aquatic ecosystems, the intimate relationships between microorganisms and other biota and the constant flow of water through the gastrointestinal tract of fish and invertebrates will also affect their indigenous microbiota. Against this background, we may assume that the natural microbiota on eggs, larvae, fry, juveniles and adult fish may help to protect against colonization by a harmful microbiota. Another aspect seen from a microbial point of view is the fact that the microbiota of intensive rearing systems differs dramatically from that in the sea, and it is influenced by many factors such as; rearing techniques, nutrient, disinfection techniques and the use of antibiotics.

In order to adhere successfully, colonize and produce disease the pathogen must overcome the host defence system. It is well known that stress from environmental factors, such as oxygen tension, water temperature and water salinity, are important in increasing the susceptibility of fish to microbial pathogens. The water milieu can also facilitate transmission of these pathogens.

The pathogenesis of *Vibrio* infections in mammals is primarily a gut infection, and it is therefore logical to ask whether the same is true in fish. Fish pathogenic bacteria, such as *Vibrio salmonicida* and *V. anguillarum*, have been shown *in vivo* to adhere to the intestinal epithelium of fish larvae and to promote severe destruction of microvilli (Olafsen and Hansen, unpublished results, cited in Knudsen *et al*., 1999). Severe damage with loss of cellular integrity was also noted in midgut of spotted wolffish (*Anarhichas minor* Olafsen) fry infected by V. *anguillarum* (Ringø, Mikkelsen and Myklebust, unpublished data, cited in Ringø *et al*., 2002a) (Fig. 1), compared to normal enterocytes (Fig. 2) (Olsen, Myklebust and Ringø, unpublished data). Scanning electron microscopy investigations of human intestinal mucosa infected with enteropathogenic *Escherichia coli* (EPEC), showed that EPEC adhere intimately in microcolonies and cause gross alterations at the apical surface of infected enterocytes (Knutton *et al*., 1987; Knutton, 1995).

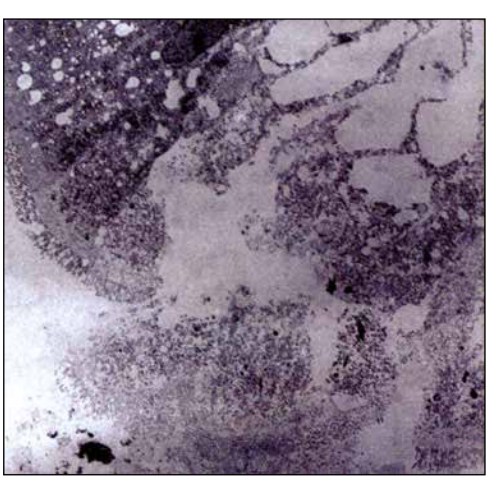

Figure 1. Spotted wolffish (*Anarhichas minor* Olafsen) fry infected by *Vibrio anguillarum*. Notice the severe cellular damage. (after Ringø, Mikkelsen and Myklebust, unpublished data)

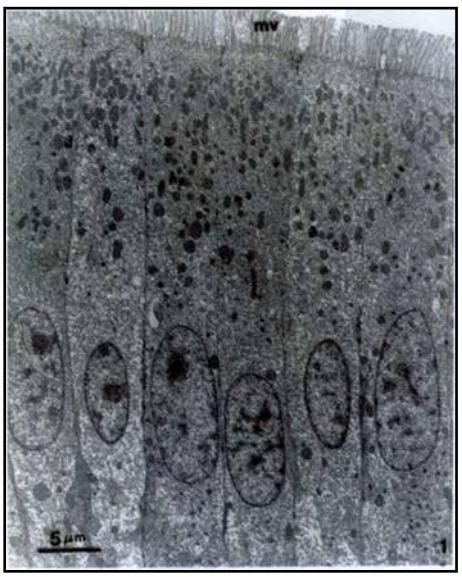

Figure 2. Normal enterocytes. Cells display the normal columnar arrangement with intact intracellular junctions and a regular, well-defined microvillous brush border (mv) at the cell apex. (after Olsen, Myklebust and Ringø, unpublished data)

The susceptibility of early life stages of turbot and Atlantic halibut (*Hippoglossus hippoglossus* L.) to *Aeromonas salmonicida* ssp. *salmonicida* was studied in challenge experiments (Bergh *et al*., 1997). Larvae of both species experienced high mortality during the yolk sac stage, and the authors suggested that this was as a result of the challenge test. However, the bacterium could not be recovered from the larvae by culture, but the pathogen was shown to be present in the intestinal lumen of some turbot larvae examined using immunohistochemical techniques (Fig. 3). Based on this result, the authors (Bergh *et al*., 1997) proposed that A. *salmonicida* ssp. *salmonicida* may persist in the larvae.

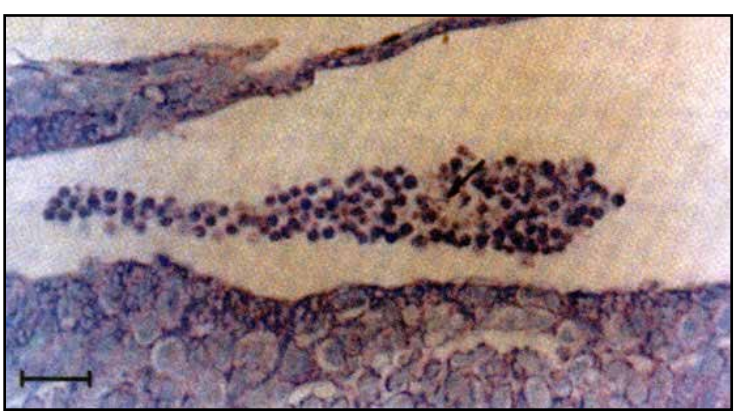

Figure 3. Semithin sections from turbot (*Scophthalmus maximus L.*) larva of group As-A, challenged with *Aeromonas salmonicida* ssp. *salmonicida* stained with Giemsa. Cell-like structures can be seen associated with positively red-stained bacteria-like structures (arrow). Scale bar is 10 µm. (after Bergh *et al*., 1997)

Endocytosis of bacteria by enterocytes has been observed in the hindgut of several fish species (Ringø *et al*., 2002a). In their study on turbot (*Scophthalmus maximus* L.) larvae, Grisez *et al.* (1996) reported free *V. anguillarum* from an endosome in the lamina propria by immunohistochemical staining (Fig. 4).

Figure 4. *Vibrio anguillarum* infection in turbot (*Scophthalmus maximus L.*) after oral challenge. *Vibrio anguillarum* attached to the microvilli (brush border) of the intestinal epithelium. (after Grisez *et al*., 1996)

Readers with special interest in pathogenesis and the gastrointestinal tract of growing fish are referred to the review of Birkbeck and Ringø (2002).

PROTECTION AGAINST BACTERIAL ADHESION MUCUS

The internal surface of the host is the first defence barrier to infection. Intestinal mucins secreted by specialized epithelial goblet cells located in the intestinal enterocytes form a viscous, hydrated blanket on the surface of the intestinal mucosa that protects the delicate columnar epithelium. This is thought to be a vital component of the intestinal mucosal barrier in prevention of colonization by pathogens in both fish and endothermic animals (Florey, 1962; Forstner, 1978; Westerdahl *et al*., 1991; Maxson *et al*., 1994; Mims *et al*., 1995; Henderson *et al*., 1999). Gastrointestinal mucus is thought to have three major functions: (1) protection of the underlying mucosa from chemical and physical damage; (2) lubrication of the mucosal surface; and (3) to provide a barrier against enteroadherence of pathogenic organisms to the underlying mucosal epithelium.

It is well known that intestinal mucus is composed almost entirely of water (90-95%) and the electrolyte composition is similar to plasma, accounting for about 1% of the mucus weight. The remaining 4-10% is composed of high-molecular-weight glycoproteins (mucins), consisting of a protein core with numerous carbohydrate (fucose and galactose) sidechains. Hydrolysis of intestinal mucus material of rainbow trout liberated increased amounts of N-acetylgalactosamine and N-acetylglucosamine (O'Toole *et al*., 1999), indicated that these carbohydrates may be present as mucin-bound moieties in fish intestinal mucus as the case for mucus from other animal species (Roussel *et al*., 1988). The majority of intestinal mucus-associated lipids in rainbow trout partitioned to the organic phase during extraction with chloroform/methanol and was found to contain

saturated and unsaturated free fatty acids, phospholipids, bile acid, cholesterol, and monoglycerides and diglycerides (O'Toole *et al.*, 1999).

Olsson *et al.* (1992) put forward the hypothesis that the gastrointestinal tract is a site of colonization of *V. anguillarum* as the pathogen could utilize diluted turbot intestinal mucus as sole nutrient source. In a later study, Garcia *et al.* (1997) concluded that Atlantic salmon (*Salmo salar* L.) intestinal mucus is an excellent growth medium of *V. anguillarum.* This result is an important aspect of the pathogenesis of this pathogen.

The mucous blanket is constantly renewed by the secretion of high molecular weight glycoproteins from individual goblet cells throughout the epithelium. Goblet cells differentiate in the lower portion of the crypts of both small and large intestine and gradually migrate onto the villi or mucosal surface.

In an early study on histopathology changes caused by *V. anguillarum*, Ransom *et al.* (1984) found large amounts of goblet (mucus producing) cells in the anterior part of gastrointestinal tract of infected chum salmon (*Oncorhynchus keta* Walbaum). The first reaction of Arctic charr (*Salvelinus alpinus* L.), a salmonid fish, infected by pathogenic bacteria (A. *salmonicida* ssp. *salmonicida*) the causative agent of furunculosis is to peel off the infected mucus by increased goblet (mucus producing) cell production compared to uninfected fish (Lødemel *et al.*, 2001) (Fig. 5). A similar reaction to that found in infected Arctic charr, is also observed in rabbit and rats infected by pathogenic bacteria (Mantle *et al.*, 1989, 1991; Enss *et al.*, 1966), and this reaction may be considered a normal host response to particular intestinal infections (Mims *et al.*, 1995).

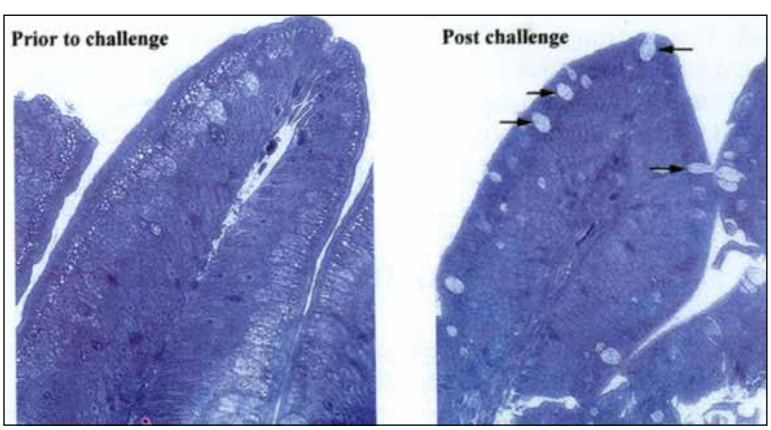

Figure 5. Light microscopic view of villi in the midgut from Arctic charr (*Salvelinus alpinus* L.) fed soybean oil prior to challenge and post challenge with the fish pathogen *Aeromonas salmonicida* ssp. *salmonicida.* Note the substantially more conspicuous goblet (mucus producing) cells (arrows) along the villi of infected fish. (after Lødemel *et al.*, 2001)

Furthermore, the peel off of mucus might lead to loss of the autochthonous (indigenous) microbiota closely associated with the intestinal epithelium forming one of the first defence to limit colonization of pathogenic bacteria.

AUTOCHTHONOUS MICROBIOTA

Savage (1983) defined bacteria isolated from the digestive tract as either autochthonous (indigenous) or allochthonous (transient). Recently, Ringø and Birkbeck (1999) presented a list of criteria (found in healthy animals, colonize early stages and persist throughout life, found in both free-living and hatchery-cultured fish, grow anaerobically, and found associated with epithelial mucosal in the digestive tract) for testing autochthony of bacteria from the gastrointestinal tract of fish. To define the presence of autochthonous microbiota in fish, electron microscope investigations are a useful tool (Ringø *et al.* 2002a). One might put forward the hypothesis that autochthonous microbiota associated closely with the intestinal epithelium form a barrier serving as the first defence to limit direct attachment or interaction of pathogenic bacteria to the mucosa as reported for endothermic animals (van der Waaij *et al*., 1972; Forstner, 1978; Slomiany *et al*., 1994; Henderson *et al*., 1996). A remarkable feature of the indigenous intestinal microbiota of fish is that situations like stress, antibiotic administration, and even small dietary changes, affect the microbial community of the digestive tract. The stability of the intestinal flora is an extremely important factor in the natural resistance of fish to infections produced by bacterial pathogens in the digestive tract. Interest in the phenomenon of resistance provided by flora components against colonization by pathogens has existed for many years in the endothermic literature (for review see Hentges, 1983; Hentges, 1992; Tancrède, 1992; Salminen *et al*., 1996). A fundamental question when discussing translocation from the gastrointestinal tract in fish is if the autochthonous microbiota has a protective role against pathogenic bacteria by producing antibacterial substances. Information about the existence of antibacterial substances produced by bacteria isolated from the digestive tract of fish has been demonstrated in several comprehensive reviews (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999; Gomez-Gill *et al*., 2000; Gram and Ringø, 2002; Ringø *et al*., 2002b). However, it is not yet known to what extent the natural microbiota of fish may be protective towards pathogen colonization. This is an important subject to clarify as the aquaculture industry is plagued by many disease problems, and an important goal for the microbiologist should therefore be to increase colonization of the gut by bacteria with an antibacterial potential against fish pathogens.

PROBIOTICS

The use of food containing live microorganisms with beneficial properties has been known for centuries. O'Sullivan *et al.* (1992) referee to Plino who advocated the use of fermented milk products in the treatment of various gastrointestinal infections as early as 76 BC. In modem time the term "probiotic" was first used in 1954 by Vergin, but since then, many different variations of the definition have been proposed (Table 1).

Antibiotic treatment to prevent infection is not recommended because of selection for strains resistant to chemotherapy but also because of concern about environmental risk and that resistant strains in the environment may transfer R plasmid to human intestinal microbiota. Nevertheless, its use is still a practical measure even methods do not prevent pathogenic proliferation in the system. Furthermore, the use of antibiotics may dramatically change the intestinal microflora of the fish and, thus impair its first-line defences (Austin and Al-Zahrani, 1988; Strøm and Ringø, 1993). An alternative method to antibiotic treatment would be the use of

Table 1. Definitions of probiotics by various authors (after Gram and Ringø, 2002)

Definition	Reference	Comments
microbial compounds which promotes body functions and beneficial microorganisms	Vergin, 1954	products - not live culture?
microbially produced "factors" which promote growth of other organisms	Lilly and Stillwell, 1965	growth promotion
animal feed supplements - organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance	Parker, 1974	only feed what is "microbial balance"
live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance	Fuller, 1989	only feed what is "microbial balance"
a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract	Naidu *el al*., 1999	only feed what is "microbial balance"
live microorganisms supplemented in food or feed which give beneficial effects on the intestinal microbial balance	Gildberg *et al.*, 1997	only feed what is "microbial balance"
a live microbial supplement which beneficially affects the host animal by improving its microbial balance	Gram *et al*., 1999	what is "microbial balance"
is based on elimination of harmful microflora from the animal's digestive tract	Bogut *et al.*, 1998	elimination? only dietary tract
mono- or mixed cultures of live microorganisms which when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora	Havenaar *et al.*, 1992 Conway, 1996 Holzapfel *et al*., 1998	what are "properties of indigenous microflora"
viable microorganisms (bacteria or yeasts) that exhibit a beneficial effect on the health of the host when they are ingested	Salminen *et al*., 1998	
beneficial bacteria which may override pathogens by producing inhibitory substances, or by preventing pathogenic colonization of the host	Riquelme *et al.*, 2000	what is "override"
beneficial bacteria that displace pathogens by competitive processes or by release of growth inhibitors	Moriarty, 1997	
live intestinal bacteria that are added to promote the viability of the host, but the term is also proper for bacteria able to regulate colonization of the outer surfaces	Skjermo and Vadstein, 1999	how is "colonization" regulated?
live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment	Verschuere *et al*., 2000	
oral probiotics are living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition	Guarner and Schaafsma, 1998	
live microbial cultures added to feed or environment (water) to increase viability (survival) of the host	Gram and Ringø, 2002	

probiotics, or beneficial bacteria, which override pathogens by producing inhibitory substances, or by preventing pathogenic colonization in the host.

Table 2 shows the effect of addition of probiotic microorganisms (*Vibrio pelagius*, *V. mediterranei* Q40, *Aeromonas media*, *Pseudomonas* ssp. *Vibrio* ssp. and *Thalassobacter utilis*) on fish and crustacean larval survival.

Table 2. Effect of addition of probiotic microorganisms on fish and crustacean larval survival (after Gram and Ringø, 2002)

Presumed probiont	Pathogen	Host organisms	Effect on survival	Reference
Vibrio pelagius	not known	Turbot larvae	Increase accumulated survival from 6-9% on day 12 and from day 0-3% on 16 after hatching	Ringø and Vadstein, 1998
Vibrio mediterranei Q40	not known	Turbot larvae	Increase accumulated survival (5 days post hatching) in 5 separate experiments (*e.g.* 14 to 55% in trial lor 75 to 81% in trial 4).	Huys *et al*., 2001
Aeromonas media	*Vibrio tubiashii*	Oyster larvae	Increase survival after 6 days from 4-100%	Gibson *et al*., 1998
Pseudomonas and unknown strain	*Vibrio anguillarum* like	Scallop larvae	Increase survival from 5-60% after 14 days	Riquelme *et al*., 1997
Pseudomonas and *Vibrio*	field trial? not known	Scallop larvae	Same survival after 48 hours as antibiotic treated tanks	Riquelme *et al*., 2001
Thalassobacter utilis	field trial not known (*Vibrio* spp.)	Crab larvae	Increase survival from 16 to 26%	Nogami and Maeda, 1992 Nogami *et al*., 1997

Gatesoupe (1997) tested in his study a siderophore-producing *Vibrio* as probiotic by feeding infected turbot larvae with rotifers enriched by the *Vibrio.* He reported improved larval survival 48 hours after infection. However, 10 days post-infection, no difference was seen in survival. In contrast to these results, Ringø and Vadstein (1998) reported that addition of *V. pelagius*, originally isolated from turbot larvae, to early developing turbot larvae had no short term effect, but caused a slight increase in survival after 12-16 days compared to larvae exposed to *Aeromonas caviae.* In an earlier study, Ringø *et al.* (1996) suggested by using an enzyme-linked immunosorbent assay that *V. pelagius* seems to colonize the larval gut when the bacteria was added to the tank water at the day of hatching but it was, however, not shown whether the

bacterium could persist in the gut if the larvae were removed from the *V. pelagius*-containing tank water.

In a recent study, Huys *et al.* (2001) searched for beneficial bacterial strains for turbot larviculture and reported increased survival of larvae by addition of a *V. mediterranei* Q40 originally isolated from sea bream larvae and an unknown organism isolated from turbot larvae at a concentration of 10^5 bacteria per ml water.

Some information is available on bacterial probiotics in culturing of Pacific oyster (*Crassostrea gigas* Thunberg) larvae. Gibson *et al.* (1998) used an *Aeromonas* media-like strain, originally isolated from Koi carp (*Cyprinus carpio*) from the Hawksbury River (Gibson, pers. com., 2001 to Lone Gram, Danish Institute for Fisheries Research, Denmark), by adding the probiont to waters of oyster larvae which had been challenged with the pathogen *V. tubiashii.* The challenge test caused an increase in numbers of the pathogen and a complete kill of the oyster population within five days, but by adding the *Aeromonas* media strain together with the pathogen, reduced numbers of *V. tubiashii* and resulted in complete survival of the population. These results are in accordance with earlier results demonstrating that additions of both algae (McCausland *et al*., 1999) and bacteria (strain CA2) (Douillet and Langdon, 1994) improved growth of the Pacific oyster larvae.

Riquelme *et al.* (1997) investigated 506 bacterial isolates, obtained from laboratory and hatchery sources for their potential probiotic effect in Chilean scallop (*Argopecten purpuratus* Lamarck 1819) larval culture. Initially, both a *Pseudomonas* isolate showing *in vitro* activity against a larval pathogen a *V. anguillarum*-related strain and an unidentified isolate with no *in vitro* inhibitory activity were found to improve survival from 5% in the non-probiotic treated to 60% in the probiotic treated over a 14 day period. Screening the 506 bacterial strains, the authors found that only 11 isolates were able to inhibit growth of a *V. anguillarum* related bacterium associated with mortality of scallop larvae. However, several strains showing *in vitro* activity increased mortality of scallop larvae. Thus, this study clearly demonstrates the importance of *in vivo* testing, as strains with *in vitro* effect may be dangerous to the animals, and strains with no *in vitro* effect may have probiotic effects *in vivo.*

Recently, Riquelme *et al.* (2001) reported that growth and survival in field trials with Chilean scallop larvae treated with pathogen-antagonizing bacteria (*Vibrio* sp. C33 and *Pseudomonas* sp. 11) at 10^3 colony forming units per ml (CFU/ml) were the same as when the larvae were treated with antibiotics. The antagonizing bacteria were added to the water at the initiation of the experiment and again after 48 hours. Controls with no treatment were not included as the commercial producer experienced rapid mortality when no treatment was used. In an earlier investigation, Riquelme *et al.* (2000) studied the uptake of pathogen-inhibiting bacterial cultures in Chilean scallop larvae, and found that an *Arthrobacter* was ingested in significant numbers. This can be a way of continuously adding the probiotic culture to the scallop larvae. The *Arthrobacter* strain was not tested in *in vivo* infection trials.

In two studies, Nogami and Maeda (1992) and Nogami *et al.* (1997) added 10^5-10^6 CFU/ml of bacterial culture isolated from shrimp pond to seawater used for crab (*Portunus trituberculatus*) culture. The strain was a Gram-negative, non-fermentative, motile rod identified as *Thalassobacter utilis* (Maeda and Liao, 1992; Nogami *et al*., 1997). The culture, which was added once every

seven days, was selected based on its ability to improve survival in *in vivo* infection trials. The organism also inhibited growth of *V. anguillarum*, *in vitro.* By adding the culture, a decline in concentration of *Vibrio* spp. in the seawater occurred and crab survival was significantly improved. It should be emphasized that the addition of five other microbial cultures (e.g. a *Bacillus subtilis*) accelerated mortality of the larvae (Nogami and Maeda, 1992).

Prevention of bacterial disease in growing fish is somewhat easier than in larvae, fry and juveniles. However, vaccination of fish smaller than 35 g (Intervet International, The Netherlands; Aqua Health Ltd, Canada) is not recommended, making the smaller stages of fish still susceptible to infection. For a range of bacterial pathogen-host combinations, good vaccines have been developed, and their optimization and use will be facilitated as further understanding of the pathogen virulence factors and of the host immune system emerges. The studies described in Table 3 should be regarded as trials of the probiotic concept rather than as suggestions for actual use of probiotics. Both the bacteriophage, *Tetraselmis*, Gram-positive bacteria (bacilli and carnobacteria, a lactic acid bacteria) and Gram-negative bacteria such as *Vibrio* and *Pseudomonas* have been evaluated as potential probionts and the experiments cover both additions to the rearing water or incorporation in the feed (Table 3).

The pathogens which are most frequently used include; *Pseudomonas plecoglossicida*, *anguillarum*, *V. ordalli*, *Yersinia ruckeri*, and *A. salmonicida*. In most of the cited studies in Table 3 increased survival of the host organisms were observed.

Readers who want more information about probiotics in aquaculture are referred to the reviews of Gatesoupe (1999), Gomez-Gil *et al.* (2000), Verschuere *et al.* (2000) and Gram and Ringø (2002).

LACTIC ACID BACTERIA (LAB)

It is suggested that lactic acid bacteria (LAB) along with other bacteria that belong to the autochthonous (indigenous) microbiota of aquatic animals might be an important part of the defence mechanism against colonization of fish pathogens in the gastro-intestinal tract. In addition to the antagonistic microorganisms colonizing the mucus surface in the natural microbial defence mechanisms, it has been shown that the surface mucus also plays a role in the prevention of colonization by parasites, bacteria and fungi.

Effects of LAB administration on intestinal microbiota

It is well known that LAB under normal circumstances are not numerically dominant in the digestive tract of fish (Ringø and Gatesoupe, 1998). In order to increase the proportion of LAB, some investigations have attempted to increase their population level by dietary factors such as: (1) chromic oxide (Ringø, 1993); (2) different oils (Ringø *et al.*, 1998; Ringø *et al.*, 2002b); (3) high and low dietary lipids (Ringø and Olsen, 1999); and (4) inulin (Ringø, Myklebust and Olsen, unpublished results). Another important criterion for the use of LAB in commercial aquaculture, is the colonization potential of LAB in the fish gut, as Vibrionaceae may also persist

Table 3. Effect of addition of probiotic microorganisms on fish survival. (after Gram and Ringø, 2002)

Presumed probiont	Pathogen	Host organisms	Effect on survival	Reference
bacteriophage[1]	*Pseudomonas plecoglossicida*	ayu	increase survival from 35-75%	Park *et al*., 2000
Tetraselmis suecica	Several Gram-negatives	salmon	increase survival from 0-15% to 20-100%	Austin *et al*., 1992
Bacillus spp.	field trial not known	channel catfish	increase survival from 56-80%	Queiroz and Boyd, 1998
Carnobacterium sp.	*Vibrio anguillarum*	salmon	no effect on survival	Robertson *et al*., 2000
	Vibrio ordalii		increase survival from 23-74%	
	Yersinia ruckeri		increase survival from 42-71%	
	Aeromonas salmonicida		increase survival from 0-20%	
	Aeromonas salmonicida	trout	increase survival from 32-74%	
Vibrio alginolyticus	*Aeromonas salmonicida*	salmon	increase survival from 0-82%	Austin *et al*., 1995
	Vibrio anguillarum		increase survival from 10-26%	
	Vibrio ordalii		increase survival from 0-26%	
	Yersinia ruckerii		no effect on survival	
Pseudomonas fluorescens	*Aeromonas salmonicida*	salmon	increase healthy fish from 25-70 to 90-100%	Smith and Davey, 1993
Pseudomonas fluorescens	*Vibrio anguillarum*	trout	increase survival from 50-70%	Gram *et al.*, 1999
Pseudomonas spp.	*Vibrio anguillarum*	trout	increase survival (some strains) no effect (some strains)	Spanggaard *et al.*, 2001
Pseudomonas fluorescens	*Aeromonas salmonicida*	salmon	no effect on survival	Gram *et al.*, 2001

[1] It is debatable whether or not a bacteriophage qualifies as a probiont "a live microbial...." due to the inert nature of viruses

for days or weeks in fish (Austin *et al*., 1995; Munro *et al*., 1995; Ringø and Vadstein, 1998). Some recent studies have demonstrated that carnobacteria strains are able to survive for several days in the intestine of larval and juvenile fish (Strøm and Ringø, 1993; Jöborn *et al*., 1997; Gildberg and Mikkelsen, 1998; Ringø, 1999). Three of these studies (Jöborn *et al*., 1997; Gildberg and Mikkelsen, 1998; Ringø, 1999) have suggested that there is apparently no host specificity with regard to colonization of the fish gut with carnobacteria, contrary to endothermic animals where adhesion of LAB appears to be complicated by host specificity (Lin and Savage, 1984; Fuller, 1986, 1989; Conway, 1989). However, the colonization site in the fish gut is also an important criterion. In a recent study, Gildberg and Mikkelsen (1998) administered two *Carnobacterium divergens* strains originally isolated from the intestine of mature Atlantic cod (*Gadus morhua* L.) and Atlantic salmon, to Atlantic cod juveniles via the food. When the Atlantic cod isolate was used, the authors only detected *C. divergens* in pyloric caeca, while the concentration of the bacteria was approximately ten fold higher in the pyloric caeca than in the intestine when the salmon isolate was used.

Transient bacteria may also be efficient if the cells are introduced at high dose. Moreover, as LAB may exert antibacterial effects against undesirable microbes, some investigators have attempted to increase the proportion of LAB associated with the fish digestive tract. In a study with four days old Atlantic cod larvae, Strøm and Ringø (1993) used an antagonistic LAB strain which, when added to the rearing water, favourably influenced the intestinal microbiota of the larvae by increasing the proportion of LAB from approximately 5% up to 70% and by a subsequent decrease in the proportion of the bacteria genera *Pseudomonas*, Cytophaga/*Flexibacter* and *Aeromonas* (Table 4). These results indicate that the LAB are able to colonize and may comprise a major part of the autochthonous microbiota in the gut of the larvae. A similar increase in intestinal LAB was also found in Atlantic cod fry fed a diet containing C. *divergens* (Gildberg *et al*., 1997) (Table 4). In a study with Atlantic salmon fry, Gildberg *et al.* (1995) demonstrated that administration of LAB reported as *Lb. plantarum*, but later reclassified as *C. divergens* (Ringø *et al*., 2001a) increased the proportion of adherent LAB to intestinal wall from nil to 100% (Table 4).

Recently, Byun *et al.* (1997) evaluated the effect of LAB (*Lactobacillus* sp. DS-12) administration via the feed on the intestinal microbiota of flounder (*Paralichthys olivaceus)* after one month of feeding (Table 4). *Lactobacillus* sp. DS-12 was not detected in the intestine of the control group, but 10^7/g LAB were found in the GIT when the fish were fed a LAB supplemented feed.

In a recent study, Bogut *et al.* (2000) evaluated the effect of *Enterococcus faecium* on the intestinal microbiota of Sheat fish (*Silurus glanis*). In this study, the fish were exposed to *E. faecium* by including the bacteria in the diet. After approximately two months of feeding, some interesting differences in the intestinal microbiota were observed between the two rearing groups. *Enterococcus faecium*-administration decreased the population level of *Staphylococcus aureus*, *Escherichia coli* and other bacteria of the family Enterobacteriaceae, and resulted in complete elimination of *Clostridium* spp. (Table 4).

Only one investigation has evaluated the influence of a commercial LAB preparation on the allochtonous intestinal microbiota. Supplementation of one gram of *E. faecium* M74 per 100 kg feed influenced the intestinal microbiota of 0^+ Israeli carp (*Cyprinus carpio*) to some extent (Bogut *et al*., 1998). While *E. coli* disappeared from the intestinal microbiota of the fish after 14

Table 4. Effect of lactic acid bacteria (LAB) administration on intestinal microbiota. (after Ringø *et al*., 2002)

Fish species	LAB used	Bacterial genera isolated and proportion of microflora population before administration (control)	after administration	after challenge	References
Atlantic cod - larvae	*C. divergens*	*Pseudomonas* 42.5; Cytophaga/ Flexibacter 42.5 *Aeromonas* 10; *C. divergens* 5	*C. divergens* 70; *Pseudomonas* 20	*	Strøm and Ringø, 1993
Atlantic cod - fry	*C. divergens*	No information was given	No information was given	*C. divergens* 75 *Pseudomonas-like* 25	Gilberg *et al*., 1997
Atlantic salmon-fry	*C. divergens*	*Pseudomonas*, Enterobacteriaceae Gram positive cocci	*C. divergens* 100	*A. salmonicida* 90 *C. divergens* 10	Gildberg *et al*., 1995
Turbot - larvae	*C. divergens*	*C. divergens* n.d.	*C. divergens* ($8x10^3$)	*	Ringø, 1999
Flounder[1]	*Lactobacillus* sp.	Enterobacteriaceae 4.3 (5/5); G(+) 4.6 (5/5)	Enterobacteriaceae 4.8 (5/5); G(+) 4.3 (5/5)	*	Byun *et al*., 1997
	DS-12	Yeast 4.6 (5/5); Hemolytic bacteria 5.8 (2/5) Mucoid colony form 4.8 (1/5); Aerobes 8.5 (5/5) Anaerobes 7.6 (5/5)	*Lactobacillus* sp. DS-12 7.0 (3/5) *Clostridium* 4.3 (1/5); Yeast 4.3 (1/5) Hemolytic bacteria 5.1 (1/5)		
		Aerobes 7.3 (5/5); Anaerobes 6.6 (5/5)			
Carp[b]	*Ent. faecium*	Enterobacteriaceae 6.2; *E. coli* 4.2 *Ent. faecalis* 3.3; *Staph. aureus* 3.7 *Bacillus* spp. 7.0; *Clostridium* spp. 2.9	Enterobacteriaceae 6.2; *E. coli* n.d. *Ent. faecalis* 3.5; *Staph. aureus* 4.0 *Bacillus* spp. 7.0; *Clostridium* spp. 2.7	*	Bogut *et al*., 1998
Sheat fish[c]	*Ent. faecium*	*Escherichia coli* 3.1; Enterobacteriaceae 3.0 *Staph. aureus* 4.7 *Bacillus* 6.0; *Clostridium* 2.1	*Escherichia coli* 1.1; Enterobacteriaceae 1.9 *Staph. aureus* 1.4 *Bacillus* 5.6; *Clostridium* n.d	*	Bogut *et al*., 2000

[a] - Data are presented as log 10 and frequency are shown in parentheses; [b] - Data are presented as log 10 after 4 weeks of feeding
[c] - Data are presented as log 10 after 58 days of feeding; n.d - not detected; * - challenge test not done

days and onwards by feeding the probiotic preparation (Table 4), the population level of Enterobacteriaceae, *E. faecalis*, *S. aureus*, *Bacillus* spp. and *Clostridium* spp. were not reduced as a result of including *E. faecium* into the diet (Bogut *et al*., 1998). The authors suggested a high adhesive ability in the epithelium of carp digestive tract for *E. faecium.* However, as they isolated the allochthonous (transient) intestinal microbiota, convincing experimental evidence was not provided.

When dealing with the potential of probiotics (for example LAB) in aquaculture the fundamental question arises whether it is possible to colonize and maintain the probiotic bacteria within the digestive tract. This is particularly important when long-term exposure may be required for the probiotic effect. In this respect, electron microscope investigations are a useful tool (Ringø *et al*., 2001b, 2002a).

During the last decade some reports have been published on the nutritional contribution of LAB to the production rate of rotifer *Brachionus plicatilis* (Gatesoupe, 1990; Gatesoupe, 1991), while the control of the microbiota of rotifer cultures has received less attention.

Challenges in vivo

The major factors involved in the biocontrol of bacterial pathogens in the gastrointestinal tract are primarily those regulating the composition, functions and interactions of indigenous microbial populations with the animal tissues. This concept is supported by repeated observations that strains of transient enteropathogens can colonize intestinal habitats of endothermic animals. The fact that fish contain intestinal microbiota with antagonistic effects against fish pathogens has prompted investigators to conduct challenge experiments with LAB during the last decade (Gatesoupe, 1994; Gildberg *et al*., 1995, 1997; Gildberg and Mikkelsen, 1998; Harzevili *et al*., 1998). However, in these studies some conflicting results on the mortality were reported when the control group was compared with probiotic treatment (Table 5).

Gatesoupe (1994) suggested that *in vivo* experiments with turbot larvae using rotifers grown on LAB strains (resembling those of *Lactobacillus plantarum* or *Carnobacterium* sp.) improved the disease resistance in challenge tests with pathogenic *Vibrio* (*V. splendidus* strain VS 11). However, the results reported in this study were registered after 48 and 72 hours, beyond which the mortality pattern was not discussed. In three papers, Gildberg and Mikkelsen (1998) and Gildberg *et al.* (1995; 1997) have used two LAB strains originally isolated from Atlantic salmon and Atlantic cod by Strøm (1988). These two isolates were recently identified by 16S rDNA and AFLP™ fingerprinting as *C. divergens* (Ringø *et al*., 2001a). In challenge trials with cohabitants with *A. salmonicida*, Gildberg *et al.* (1995) in contrast to the expectations, registered highest mortality of Atlantic salmon fry with fish given the diet containing *C. divergens*, originally isolated from Atlantic salmon intestine. In their study with Atlantic cod fry, Gildberg and Mikkelsen (1998) observed the same cumulative mortality independent whether the *C. divergens* isolates supplemented to the commercial feed were originally isolated from the digestive tract of Atlantic cod or Atlantic salmon, when the fish were bath exposed to *V. anguillarum.* On the other hand, an improved disease resistance of Atlantic cod fry was observed by supplementing a commercially dry feed with a strain of *C. divergens* originally isolated from the cod (Gildberg *et al*., 1997). The

Table 5. Challenge tests of fish with lactic acid bacteria (LAB) (after Ringø *et al*., 2002)

LAB isolate used	Host	Pathogen	Way of administration	Effect in challenge test	Suggested mode of action	Reference
Carnobacterium spp.[a]	Turbot - larvae	*V. splendidus*	Enrichment of rotifers	+	Antagonism and/or improved nutritional value of the rotifers	Gatesoupe, 1994
C. divergens[b]	Atlantic salmon - fry	*A. salmonicida*	Addition to the diet	-		Gildberg *et al.*, 1995
C. divergens[c]	Atlantic cod - juveniles	*V. anguillarum*	Addition to the diet	+	Not specified	Gildberg *et al*., 1997
C. divergens[b]	Atlantic cod - fry	*V. anguillarum*	Addition to the diet	+[e]	Antagonism	Gildberg and Mikkelsen, 1998
C. divergens[c]	Atlantic cod - fry	*V. anguillarum*	Addition to the diet	-		Gildberg and Mikkelsen, 1998
Lb. rhamnosus[d]	Rainbow trout	*A. salmonicida*	Addition to the diet	+		Nikoskelainen *et al.*, 2001

+ improved disease resistance; - no significant effect

* - isolated from rotifer; [b]- isolated from intestine of Atlantic salmon (Strøm, 1988); [c] - isolated from intestine of Atlantic cod (Strøm, 1988); [d] - a probiotic for human use

[e] - Twelve days after infection significant reduced cumulative mortality was recorded in fish given feed supplemented with *C. divergens* isolated from Atlantic salmon, but no effect was detected four weeks after infection

explanation for these conflicting results has not been elucidated. Gildberg and Mikkelsen (1998) put forward a hypothesis that bacteriocin production can be inducible and may not occur if the bacteria are not frequently challenged with inhibitors as previously demonstrated by Schrøder *et al*. (1980). Furthermore, a recent study by Nikoskelainen *et al.* (2001) used the human probiotic *Lactobacillus rhamnosus* in a challenge test with *A. salmonicida* with promising results (Table 5). These results should stimulate fish microbiologist to use human probiotic LAB in future studies.

If the intestine is involved in infection as suggested by several authors, the fundamental question arises whether there are differences between the posterior part of the intestine and the hindgut region of the intestine? It is well established that the intestine in an immature or inflammatory state has an enhanced capacity to absorb intact macromolecules (for review see Olsen and Ringø, 1997). Furthermore, some studies report endocytosis of bacteria by enterocytes in the epithelial border of hindgut of herring (*Clupea harengus*) larvae (Hansen *et al*., 1992; Hansen and Olafsen, 1999), herring and Atlantic cod larvae (Olafsen and Hansen, 1992) and 36 days old juvenile turbot (Grisez *et al*., 1996). It is generally accepted that mature and non-inflammatory intestines of adult salmonids are not permeable to microparticulates in contrast to the mammalian gastrointestinal tract where M-cells are active in phagocytosis. However, a recent study demonstrated endocytosis of bacteria by enterocytes in the epithelial border of hindgut of adult salmonid fish (Fig. 6a), as well as in the posterior part of the intestine (pyloric caeca) (Fig. 6b) (Ringø *et al*., 2002b). These results are in accordance with observations made by Vigneulle and Laurencin (1991) and Tamura *et al.* (1993) who measured phagocytosis of fixed *V. anguillarum* in the posterior intestine of rainbow trout (*Oncorhynchus mykiss*), sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*) and eel (*Anguilla anguilla*).

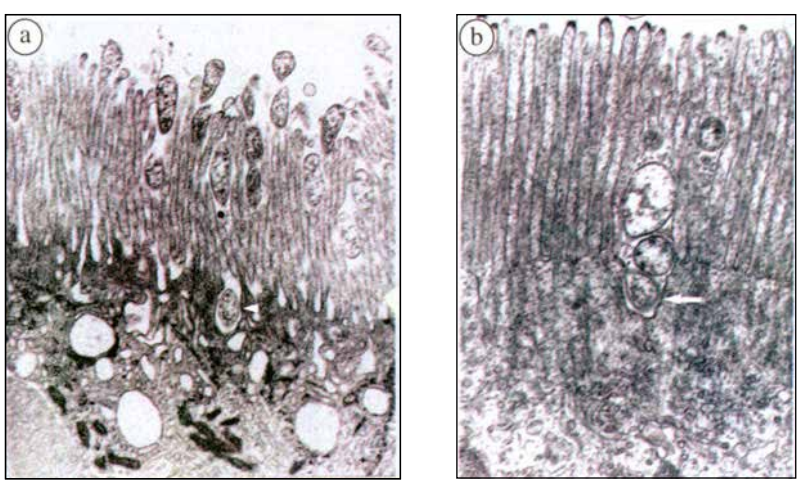

Figure 6. Endocytosis of bacteria demonstrated in the hindgut region (a) and pyloric caeca (b) of Arctic charr (*Salvelinus alpinus* L.). (after Ringø *et al*., 2002b)

The observations of Vigneulle and Laurencin (1991), Tamura *et al.* (1993) and Ringø *et al.* (2001b, 2002a, 2002b) indicate that the intestine is involved in bacterial translocation. Yet no clear evidence is available on possible differences between different parts of the intestine with regard to bacterial infection.

It is well known that rotifers are often suspected of being a vector for bacterial infections to the predating organisms (Perez-Benavente and Gatesoupe, 1988; Tanasomwang and Muroga, 1988; Nicolas *et al*., 1989). It is therefore surprising that studies dealing with the proliferation of larval pathogens in rotifer cultures are so scarce (Gatesoupe, 1991; Hazevili *et al*., 1998). Gatesoupe (1991) reported that the proliferation of *A. salmonicida* that accidentally appeared in the experimental rotifer culture was inhibited by treatment with *Lb. plantarum.* Hazevili *et al.* (1998) reported that administration of the probiotic strain *Lac. lactis* AR21 under sub-optimal feeding regime, counteracted the growth inhibition of the rotifers due to *V. anguillarum.*

Readers with special interest in lactic acid bacteria in fish are referred to the comprehensive reviews of Ringø and Gatesoupe (1998) and Ringø *et al*., 2002c.

PREBIOTICS

Specific bacterial pathogens can be an important cause of mortality as intensive husbandry practices often result in breakdown of natural barriers between the host and pathogens. Nowadays, the prevention and control of these diseases has concentrated on good husbandry practices and the use of vaccines and antibiotics. However, treatment by feeding antibiotics may cause the development of resistant bacteria through plasmids or bacteriophages (Towner, 1995). Therefore, there is an increased interest within the aquaculture industry in the control or elimination of antimicrobial use. Alternative methods need to be developed to maintain a healthy microbial environment. Two such methods that are gaining acceptance within the industry are the use of probiotic bacteria or prebiotics to control potential pathogens.

During the last decade, several reviews have dealt with the potential of probionts in aquaculture (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999; Gomez-Gill *et al*., 2000; Verschuere *et al*., 2000; Gram and Ringø, 2002; Ringø *et al*., 2002c). It is therefore a pertinent question whether it is possible to colonize and maintain probiotic bacteria within the digestive tract. This is particularly important when long-term exposure is required for the probiotic effect. However, to date, there is no real evidence demonstrating the preventive effect of probionts against colonization and adherence of fish pathogenic bacteria in aquaculture. The reason for this may be that the probionts used are unable to colonize the mucus layer of the digestive tract or external surfaces. Examination of adhesion has become a standard procedure for selecting new probiotic strains for human application (Salminen *et al*., 1996), but it is less common in aquaculture.

The addition of high doses of probiotic strains (for example, lactic acid bacteria) to established microbial communities of fish provoked a temporary change in the composition of the intestinal microbial community. However, within a few days after administration had stopped, the added strains showed a sharp decrease and were lost from the gastrointestinal tract (Jöborn *et al*.,

1997; Ringø and Gatesoupe, 1998). Another way to colonize and increase the population level of beneficial bacteria with antagonistic ability is the use of prebiotics. The modern concept of prebiotics implies the use of selective agents to favour growth of the protective indigenous gut microbiota. Dietary fiber is a prebiotic that belongs to the broad category of carbohydrates. Burkitt *et al.* (1972) defined dietary fiber as "*the sum of polysaccharides and lignin which are not digested by the endogenous secretions of the human gastrointestinal tract.*" They can be classified into soluble (*e.g.*, inulin and oligofructose), insoluble (*e.g.*, cellulose) or mixed (*e.g.*, bran). It is well known from endothermic investigations that dietary fibers are fermented by the anaerobic intestinal microbiota, primarily those colonizing the large intestine (Roberfroid, 1993; Gibson *et al.*, 1995; Roberfroid, 1995; Gibson, 1998; Rumessen and Gudmand-Høyer, 1998), leading to the production of lactic acid, short chain fatty acids (SCFA-acetate, propionate, and butyrate) and gases (H_2, CO_2 and CH_4) (Roberfroid, 1993) that are utilized by the host (Schneeman. 1999). Inulin, is a polydisperse carbohydrate consisting mainly of β (2→1) fructosyl-fructose links, generally referred to as fructan and is found in various edible fruits and vegetables such as wheat, onions, leeks, garlic, asparagus, artichokes and bananas (Roberfroid, 1993; Van Loo *et al.*, 1995). Although inulin is not a natural fiber in fish diet, the prebiotic potential of inulin and other dietary fibers may also have interesting applications in aquaculture. Some information is available about fermentation of inulin by fish gut microbiota, notably, *Carnobacterium piscicola* (Ringø *et al.*, 1998), *C. mobile* (Ringø and Olsen, 1999), and *Carnobacterium* spp. (Ringø and Olsen, 1999; Ringø *et al.*, 2001a). It is also known that dietary inulin resulted in damage to intestinal enterocytes of the salmonid fish Arctic charr (Fig. 7) (Olsen *et al.*, 2001) compared to normal enterocytes (Fig. 8), and that dietary inulin alters the adherent gut microbiota of Arctic charr (Ringø, unpublished results). However, the effect of dietary inulin on fish welfare is not yet known.

Figure 7. The epithelium in the hindgut of Arctic charr (*Salvelinus alpinus* L.) fed dietary inulin. The cells are highly vacuolated and many of the vacuoles have lamellar content (small arrows) which may be inulin. The apical surface of these cells shows sign of damage including loss of membrane and microvilli (large arrows). (after Olsen *et al.*, 2001)

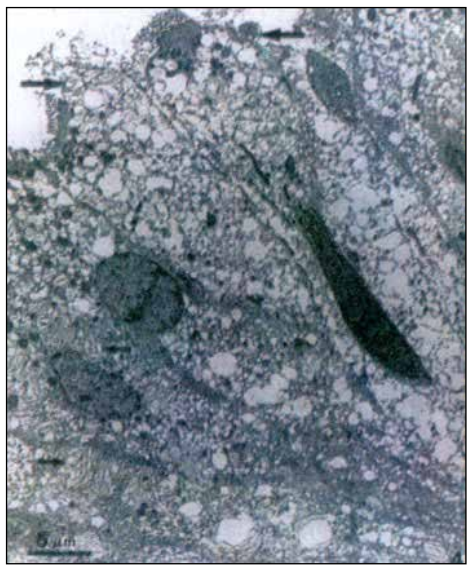

Figure 8. Epithelial cells in the hindgut of Arctic charr (*Salvelinus alpinus* L.) fed control diet (dietary dextrin). The columnar cells typically show numerous vacuoles varying in size and electron density (arrows). Cytoplasm, microvilli and intracellular organelles appear normal. In the upper middle part of the field, an effete enterocyting is probably being shed as part of normal epithelial turnover. (after Olsen *et al*., 2001)

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Selection of Probiotics for Shrimp and Crab Hatcheries

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ABSTRACT

A study aimed at obtaining a biological control agent against bacterial diseases, specifically luminescent vibriosis, of hatchery-reared shrimps and crabs was done to find an alternative for chemotherapy as a disease prevention and control method. Bacteria were isolated from crustacean rearing environments where luminescent vibriosis was not observed, from natural food, and from various feed ingredients. From hundreds of purified strains, 80 bacterial isolates were tested in one-on-one mixed cultures in seawater for their ability to suppress the growth of luminescent *Vibrio harveyi.* Of the 10 isolates exhibiting that capability, two strains were further studied: C1 from *Chlorella* culture and P9 from a commercial probiotic preparation. However, due to the indigenous nature of C1 strain from the unicellular alga *Chlorella* sp. and the ease in distinguishing it from other bacteria owing to its colony morphology, most tests were done on C1 strain. To determine the suitability of C1, and to some extent P9, as biocontrol bacteria, their pathogenicity against crab larvae and shrimp postlarvae, and their ability to become associated or incorporated into the larvae were determined. Incorporation into the rotifer, *Brachionus*, was also tested. Due to positive results obtained in the incorporation experiments, the growth of strain C1 in microbiological media and unrefined media prepared from agricultural by-products was also tested.

INTRODUCTION

The luminescent bacterium *Vibrio harveyi* is a serious pathogen in shrimp (Lavilla-Pitogo et al., 1990) and crab larval production (Fielder and Heasman, 1999). Together with cannibalism, infection with luminescent bacteria was identified as a major problem causing mortality in hatchery-reared *Scylla serrata* (Quinitio et al., 2001). The limited application of chemotherapy as an effective control measure requires the development of alternative strategies of disease control. Biological control using live bacteria may be an option for bacterial disease prevention and control in crustacean hatcheries. The mechanism involves live bacterial application to promote good health in the hosts by out-competing pathogens in the rearing environment (Nogami and Maeda 1992; Gatesoupe 1999; Skjermo and Vadstein, 1999), or improving the indigenous microflora in the gastrointestinal tract (Gildberg *et al.* 1997; Rengpipat *et al*., 1998). Probiotic application is already an accepted practice in poultry and swine industries, but there is still a need to study this approach in aquaculture (Gomez-Gil *et al*., 2000).

This project aims to test indigenous as well as commercially available bacteria as a biological control agent against microbial diseases, specifically luminescent vibriosis due to *V. harveyi*, affecting hatchery-reared crabs and shrimps. This paper describes the steps taken to find suitable bacteria and appropriate strategies for their application in crustacean larvae production systems.

BACKGROUND

The present rearing system for crab and shrimp larvae is largely based on clean rearing water in which nauplii, unicellular algae and diatoms, zooplankton and other substances are added from their respective production units (Parado-Estepa *et al*., 1996). This husbandry method creates a niche for opportunistic pathogens, specifically, bacteria. Luminescent vibriosis in larval crustaceans occurred when the hatchery system shifted from one that is ecologically balanced to one that accommodates opportunists (Lavilla-Pitogo and de la Peña, 1998).

Bacterial epizootics due to luminescent bacteria were first recognized in the mid-1980s. Outbreaks were notable because shrimp hatchery operations then had reached industry scale in producing postlarvae needed by the booming grow-out sector. Investigations showed a high incidence of luminescent vibriosis due to *V. harveyi* (Lavilla-Pitogo *et al*., 1990). Pathogenicity tests showed that exposure of *P. monodon* larvae and postlarvae to 10^2 *V. harveyi* cells/ml resulted in significant mortality within 48 h. Scanning electron microscopy also showed that infected larvae had plaques of bacteria on the mouth and feeding apparatus implying an oral route of entry for the pathogen.

Because chemotherapy induced deformities in treated larvae (Baticados *et al*., 1990) and chemicals were found generally ineffective, preventive approaches and improved hygiene in the hatchery were tried as measures to prevent disease due to luminescent bacteria in the hatchery system. The sources of luminescent *V. harveyi* in *P. monodon* hatcheries were determined from the different hatchery components (Lavilla-Pitogo *et al*., 1992) and results showed that aside from nearshore seawater used for larval rearing, spawners, whose midgut bacterial flora contained 16 to 17% luminescent vibrios of its total *Vibrio* population, are significant sources of luminescent vibrios. Interestingly, spawners have been observed to release large amounts of fecal material during spawning, thus facilitating bacterial colonization of newly spawned eggs (Lavilla-Pitogo, 1995). After determining the sources of infection, preventive measures like chlorination of seawater (Baticados and Pitogo, 1990), removal of spawners immediately after spawning (Lavilla-Pitogo *et al*., 1992), egg washing (Lio-Po *et al*., 1989), various feed sanitation procedures such as disinfection of zooplankton resting stages prior to hatching (Lio-Po *et al*., 1989), rinsing of *Artemia* nauplii and other zooplankton, and use of diatoms with inhibitory effects against vibrios (Lavilla-Pitogo *et al*., 1992; 1998) were seriously considered. The use of microbially matured seawater to select non-opportunistic bacterial flora in the water for rearing marine larvae (Skjermo *et al*., 1997) and the use of benign bacteria to compete with pathogens (Dopazo *et al*., 1988; Lemos *et al*., 1991) are techniques geared towards restoring microbial balance in the rearing system.

'PROBIOTIC' APPROACHES IN AQUACULTURE

In aquaculture, bioaugmentation, bioremediation, and probiotic application are terms that are sometimes used interchangeably. Although they are similar in their usage of microbes, they are dissimilar in their manner of application of the microbes or microbial products of choice. Following are their accepted definitions:

Bioaugmentation is the use of selected strains of microbes isolated from the environment to improve some of the processes involved in traditional waste treatment.

Bioremediation is the use of organisms to detoxify and clean up pollution. Techniques are applied in soils and aquifers to remove contaminants by biodegradation. *In situ* bioremediation is the enhancement of the catabolic activity of indigenous microorganisms by adding nutrients and, if necessary, oxygen.

Probiotics are viable monoculture or a mixed culture of organisms that are given with feed to inhabit the intestinal tract and contribute to good health by protecting against disease and providing better nutrition. A good probiotic should adhere to the lining of the gastrointestinal tract and produce substances which fight harmful organisms (Gibson and Fuller, 2000).

In the extensive review done on probiotic bacteria in aquaculture by Verschuere *et al.* (2000), a broader definition of probiotics was proposed to address the objections made on the earlier usage of the term. Thus, it was proposed that "a probiotic is a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment." Application of commercially available microbial preparations has generated interest among aquaculture practitioners as an alternative to antibiotics in disease control (Moriarty, 1998). Many bacterial products with probiotic value are in the Philippine market to provide biological remedies for environmental problems in aquaculture. The use of at least four of these products was reported by Primavera and co-workers (1993) to provide benefits like pathogen control, waste digestion, sludge clean up, and other waste management problems in shrimp grow-out culture.

Evidence for feasible microbial manipulation in the larval rearing environments of various aquatic species is growing (Dopazo *et al*., 1988; Nogami and Maeda, 1992; Austin *et al*., 1995; Garriques and Arevalo, 1995; Riquelme *et al*., 1997; Skjermo *et al*., 1997) to effectively control bacterial pathogens in crustacean hatcheries. Recent literature on microbial control against fish diseases include bacteria as probiotic for larvae of *P. monodon* (Rengpipat *et al*., 1998), microbial manipulation to sustain ecological balance in shrimp hatcheries (Lavilla-Pitogo *et al*., 1998), probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (Gildberg *et al*., 1997), siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae (Gatesoupe, 1997), and addition of inhibitor-producing bacteria against bacterial pathogens affecting mass cultures of the Chilean scallop, *Argopecten purpuratus* (Riquelme *et al*., 2000, 2001). A comprehensive review on the use of probiotics in aquaculture was done by Gatesoupe (1999), and the microbial control techniques used in intensive rearing of marine larvae were discussed by Skjermo and Vadstein (1999). An important review on the use and selection of probiotic bacteria for use in the culture of larval aquatic organisms was done by Gomez-Gil *et al*.,

(2000), focusing principally on results from commercial-scale shrimp larval rearing. These reports were the basis in drawing up criteria for selecting probiotic isolates. All throughout the study, the guidelines and dictums raised by Schisler and Slininger (1997) on microbial selection strategies that enhance the likelihood of developing commercial biological control products were considered.

CRITERIA USED IN SELECTING PROBIOTIC BACTERIA

Strain origin

Bacteria for potential use as biological control agent were isolated from crustacean rearing environments where disease did not occur using standard procedures and commercially available culture media. These sources include the hatchery's natural food cultures where *V. harveyi* did not occur but could. Other potential sources of bacteria included feed ingredients like rice bran and fish meal since earlier samples showed freshly-pelleted artificial feeds also harbored heat-resistant bacterial populations. Results from a previous study showed that wild-caught shrimp postlarvae harbored a population of bacteria in their gut that included relatively few vibrios and an insignificant number of luminous bacteria. Therefore, wild-caught postlarvae were identified as a source of bacterial strains for potential biological control against luminous vibriosis. Bacteria from commercially available probiotic products were also tested in its action against *V. harveyi.* From these sources, strains of bacteria were isolated using general culture media like nutrient agar (NA) and marine agar. From several hundreds of purified bacterial colonies, bacteria were grouped based on colony and cell morphology, oxidation-fermentation reactions and growth on selective media. The number of isolates for the competition experiments was trimmed down to 80 isolates (Table 1).

Table 1. The sources of bacterial isolates used in competition experiments

Source	**Number of isolates tested**
Crab eggs	15
Crab zoeae	3
Zooplankton	5
Cultured unicellular algae	25
Adult crab hemolymph	5
Commercial probiotics	10
Feed ingredients	10
Wild shrimp postlarvae	7
Total	**80**

Competition experiments in mixed cultures

Competition experiments between luminescent *V. harveyi* and the candidate biocontrol bacteria were done using mixed cultures in seawater following procedures modified from Lemos *et al.* (1991) and Lavilla-Pitogo *et al.* (1998). The sources of the bacteria used in competition experiments are shown in Table 1. Candidate biocontrol bacteria suppressed growth of *V. harveyi* following two general patters (Fig. 1). Of the 80 strains of bacteria tested, only 10 suppressed growth of *V. harveyi* within 24 h. Subsequent results presented here are based on further studies done on two of the 10 isolates, strains C1 from *Chlorella* sp. culture and P9 from a commercial probiotic product that suppressed growth of *V. harveyi* within 24 h. Bacteria that gave delayed or no suppressive action were discarded.

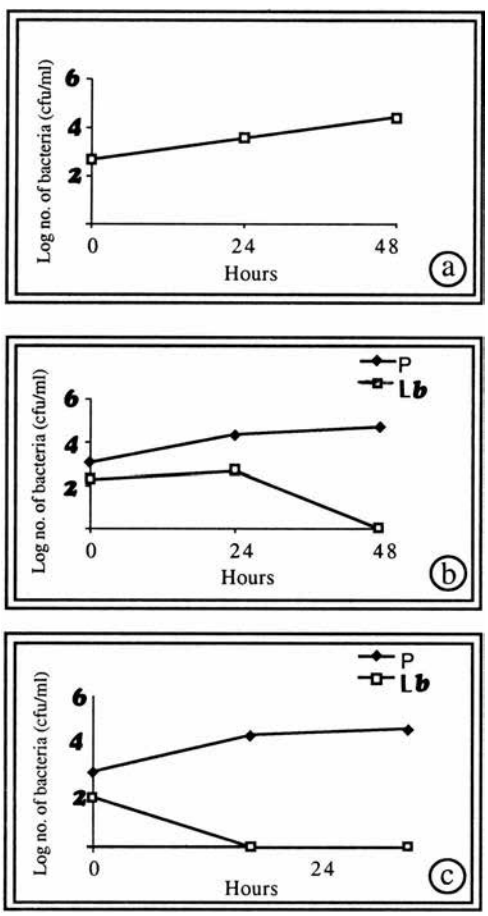

Figure 1. Patterns of bacterial growth obtained in the 48 h competition experiments: a. control - luminous bacteria only; b. inhibition of luminous bacteria after 24 h; c. inhibition of luminous bacteria within 24 h. P = candidate probiotic bacterium; Lb = luminous bacteria

Identification and detection methods

Preliminary identification of isolates was done using standard biochemical tests, although the tests did not classify the strains to genus and species. The general characteristics of isolates C1 and P9 are given in Table 2. The special characters that distinguish these isolates from other bacteria in a similar system are swarming colony for C1 and heat tolerance for P9. The swarming characteristic of strain C1 is especially important when identifying it in samples with mixed bacterial population. To control swarming, nutrient agar medium was prepared with 2% instead of 1.5% agar. The additional agar content, as well as removal of excess moisture on the agar plates by drying in an incubator, controlled the colony of C1 into 5-10 mm diameter with irregular to lobate edge.

Table 2. Characteristics of two candidate probiotic bacteria obtained from *Chlorella* sp. cultures (C1) and from a commercial probiotic (P9)

Characteristics	Bacterial Strain	
	C1	P9
Source	*Chlorella* culture	Commercial probiotic
Colony on NA*	Swarming	Large, oblate
OF* reaction	Fermentative	Oxidative
Pseudomonas - *Aeromonas* agar	Colorless	Yellow
TCBS* colony	No growth	Yellow
Special character	Swarming, fast growth	Heat tolerant

* NA = nutrient agar; OF = oxidation-fermentation; TCBS = thiosulfate citrate bile sucrose agar

Pathogenicity

In order for a bacterial strain to become an effective probiotic, it should not cause mortality to the cultured crabs or shrimps. A comparison of published information on the pathogenicity of *V. harveyi* to shrimp larvae and juveniles by static bath challenge is in Table 3. Pathogenicity tests of *V. harveyi* on various stages of crab larvae (from zoea 1 to zoea 5) showed the pathogenic level to be from 10^4 to 10^6 colony-forming-units (cfu) in static bath challenge (Zafran, unpublished). Thus, pathogenicity of the probiotic strains C1 and P9, and the luminescent *Vibrio* sp. (strain CLM3) obtained from crab larval epizootics were conducted on various stages of crab larvae and shrimp postlarvae following protocols described by Lavilla-Pitogo *et al.* (1990).

Table 3. Pathogenicity of *Vibrio harveyi* to shrimp, *Penaeus monodon*, larvae and juveniles by static bath challenge

Host	Bacterial Species	Dose/Duration	Mortality	(Reference %)/Signs
P. monodon	*V. harveyi*	10^2 - 10^3 40 - 48 h	67 - 74 = Z* 69 - 73 = M* 55 - 69 = PL*	Lavilla-Pitogo *et al*., 1990
P. monodon	*V. harveyi*	2.6 x 10^3	50 % = PL	Karunasagar *et al*., 1994
P. monodon	*V. harveyi*	<10^2 for 1 - 2 days	up to 100=Z up to 78 = M	LeGroumellec *et al*., 1995
P. monodon	*V. harveyi BP04 strain*	10^3 and 10^4 for 48 h	74.7 = Z 52.9 = M 48.5 = PL	Prayitno and Latchford, 1995
P. monodon	*V. harveyi*	10^6 - 10^7 2 h bath	bioluminescence, degeneration of hepatopancreas	Robertson *et al*., 1998
P. monodon	*V. harveyi*	2 h immersion	LD_{50} was 1.3 x 10^4 in 14 g juveniles	Saulnier *et al*., 2000

* Z = zoea; M = mysis; PL = postlarva

Figure 2 shows that the luminescent bacterium CLM3 is pathogenic to zoea 1 stage *S. serrata* larvae inducing mortality of 63 and 64% with an initial dose of 10^2 and 10^3 cfu/ml. In comparison, 40% mortality was recorded in the control. Note that although no bacteria was inoculated in the control, a mixed bacterial population of up to 10^4 cfu/ml was enumerated after 24 h. Interestingly, the figure also shows that the initial CLM3 inocula of 10^2 and 10^3 cfu/ml increased to 10^4 and 10^5 cfu/ml, respectively, after 24 h.

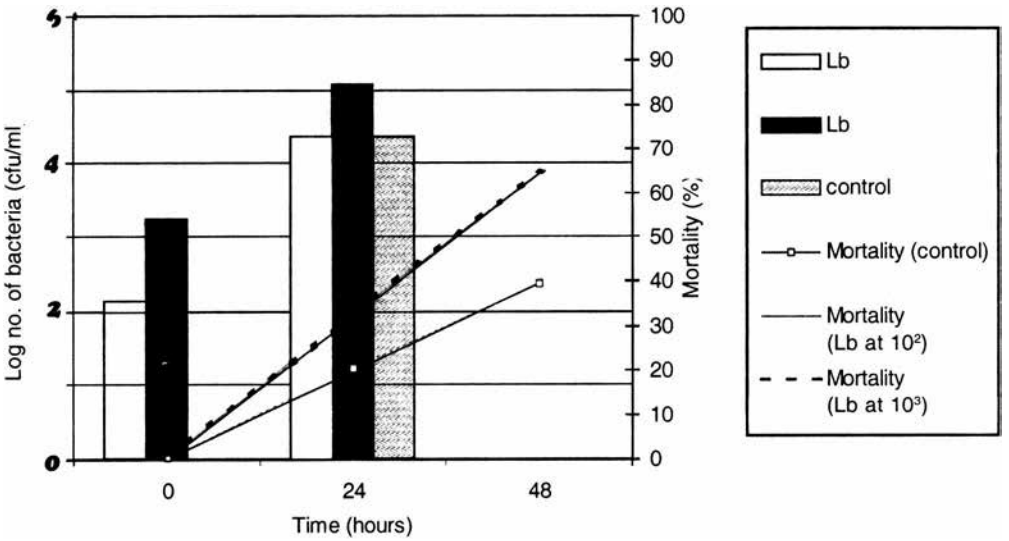

Figure 2. Pathogenicity of *Vibrio harveyi* (strain CLM3) to zoea 1 stage crab (*Scylla serrata*) larvae. Bar graphs represent CLM3 cfu/ml, while line graphs represent percentage mortality of crab larvae. Lb=luminous bacteria

Compared to the above results, Fig. 3 shows that the probiotic bacterial strains are not pathogenic because higher survival rates in crab larvae exposed to C1 and P9 were obtained even at levels of 10^5 and 10^6 cfu/ml compared with the control (no bacteria added). Crab larvae in the latter treatment succumbed to luminescent vibriosis due to contamination of test larvae. Although no bacteria were added in the control, 10^6 cfu/ml were enumerated after 24 h, 9% of which were luminescent. These results highlight the positive effect of C1 and P9 since much higher survival was obtained in those treatments.

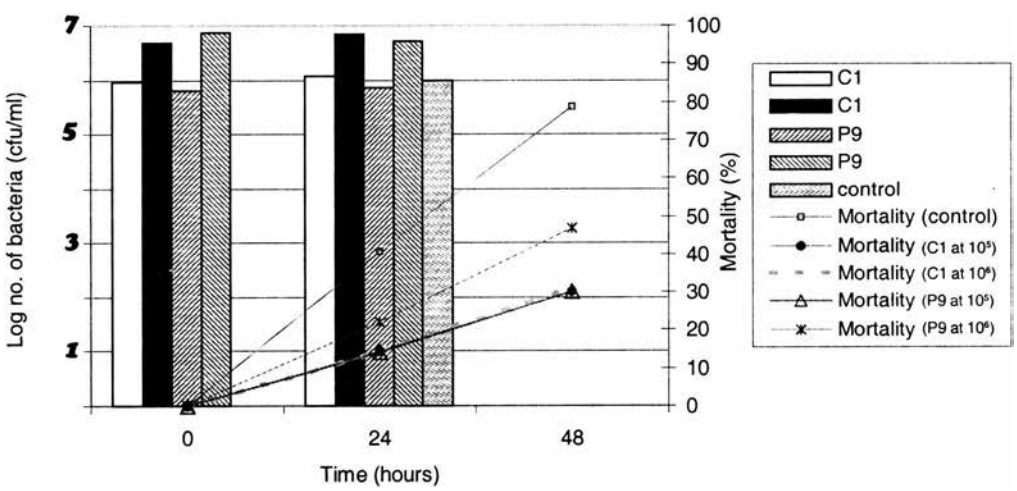

Figure 3. Pathogenicity of probiotic strains C1 and P9 to zoea 3 stage crab (*Scylla serrata*) larvae. Bar graphs represent C1 and P9 cfu/ml, while line graphs represent percentage mortality of crab larvae

Figure 4 illustrates the effect of strains C1 and P9 on shrimp postlarvae. Even at an inoculated dose of 10^5 and 10^6 cfu/ml (which increased to almost 10^6 and 10^7 cfu/ml after 24 h), mortality was 20 % or less in C1 and P9 treatments. Mortality in the control was not significantly different from those of the probiotic strains. Interestingly, the associated bacterial flora of shrimp postlarvae that had been exposed to C1 for 48h showed it to be composed mostly of C1 indicating its probiotic action. It should be noted that pathogenic levels of *V. harveyi* on larvae of *P. monodon* and *Scylla serrata* using static 48 h baths are in the range of 10^2 to 10^4 cfu/ml, which are lower than 10^5 and 10^6 cfu/ml of C1 and P9 used in this study.

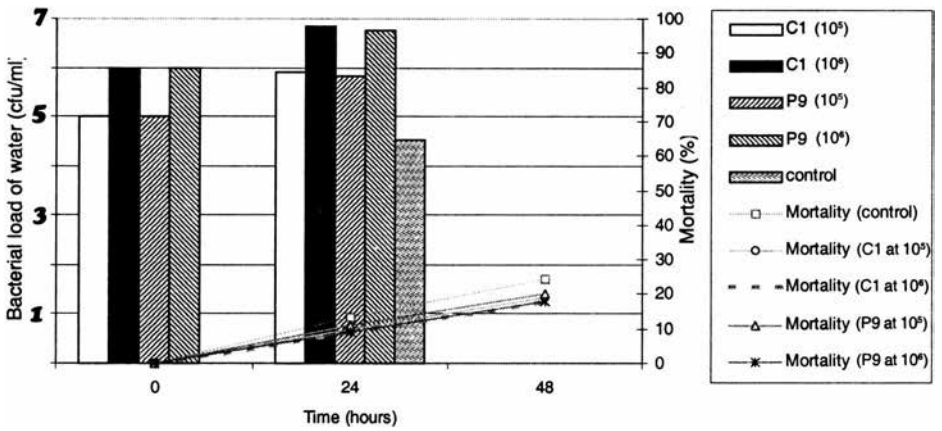

Figure 4. Pathogenicity of probiotic strains C1 and P9 to postlarva 10 stage shrimp (*Penaeus monodon*). Bar graphs represent C1 and P9 cfu/ml, while line graphs represent percentage mortality of crab larvae

TEST ON DELIVERY METHOD

Application of bacteria through live food organisms

The objective of this study was to incorporate probiotic bacterial strain C1 into live zooplankton in order to manipulate the associated bacterial flora of crustacean larvae through feeding. This was done by adding bacteria into pre-washed *Brachionus plicatilis* in sterile seawater (SSW). The animals were allowed to 'starve' in sterile seawater for 2 h after which bacterial suspensions of C1 were added to obtain a final concentration of 10^6 cfu/ml. Bacterial counts in *B. plicatilis* were done on the following periods: right after inoculation with C1 (0 h), 1 h after inoculation, and 2.5 h after inoculation. Determination of bacterial load was done by rinsing the *B. plicatilis* three times in sterile seawater. After removing excess water by blot drying on sterile absorbent paper, the animals were transferred into pre-weighed microcentrifuge tubes and homogenized. Macerated animal suspensions were serially diluted in SSW, plated on NA, Pseudomonas Aeromonas selective agar base (GSP) and thiosulfate citrate bile sucrose agar (TCBS), and incubated at 28-30 C for 18 to 24 h.

Results are presented in Fig. 5. At 0 h, no C1 colonies were associated with the animals. One h after, up to 10^8 cfu of C1/g of *B. plicatilis* was recovered. The C1 bacteria dominated over the initial bacterial population associated with the rotifers prior to inoculation (compare 0 h and 1 h bacterial loads in Fig. 5). After 2.5 h, a reduction in C1 population in the rotifers was observed proving that 1 h is enough period for incorporating C1 probiotic into live *B. plicatilis.* As live rotifers are often considered vectors for bacterial infection (Muroga *et al.*, 1987; Perez-Benavente and Gatesoupe, 1988), the successful incorporation of probiotic bacteria to eliminate potential pathogens from zooplankton or to effectively deliver beneficial bacteria into the culture system provides a window of opportunity for effective biological control. While similar approaches have been tried for *Lactococcus lactis* AR21 strain (Shiri Harzevili *et al.*, 1998) and 4:44 and PB52 strains for first feeding turbot larvae (Makridis *et al.*, 2000), there is a need to develop the technology of C1 application to crustacean larviculture.

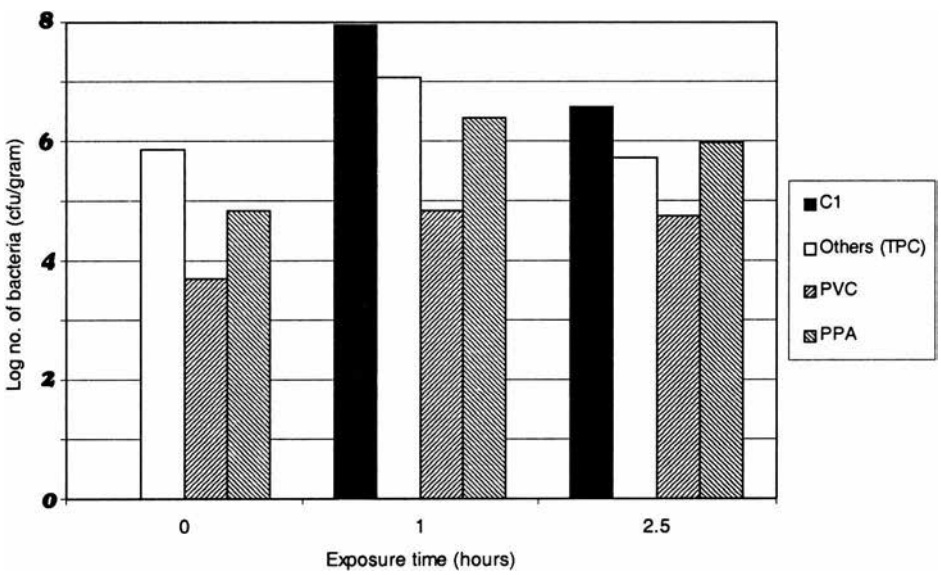

Figure 5. Results of incorporation of probiotic strain C1 into live zooplankton *Brachionus plicatilis.* TPC = total plate count in nutrient agar; PVC = presumptive *Vibrio* count in thiosulfate citrate bile sucrose agar (TCBS); PPA = presumptive *Pseudomonas* and *Aeromonas* count in GSP medium

GROWTH OF BACTERIAL STRAIN C1 IN VARIOUS LIQUID MEDIA

Aside from efficacy, an important criterion to fulfill in the search for a good bacterial probiotic is favorable growth kinetics when grown in commercially feasible liquid media (Schisler and Slininger, 1997). The growth of probiotic bacterial strain C1 in media derived from agricultural by-products like molasses, coconut cream and rice bran was tested. This information is important when large-scale production of bacteria will be needed. For comparison, microbiological grade liquid media like nutrient broth (NB) and brain heart infusion broth (BHIB) were also used to compare the peak bacterial densities obtained.

Figure 6 shows the growth curves of strain C1 in various media. Peak cell densities of 10^8 cfu/ml were obtained on Day 2 in NB and BHIB. Among the media derived from agricultural by-products, high cell densities of up to 9 x 10^7 were obtained in 10% rice bran extract (pH 7). Growth was not as profuse in crude media using 1 % coconut cream and 10% rice bran extract with unadjusted pH of 5. No growth was obtained in 1% molasses medium indicating the inability of C1 to utilize its major component, sucrose, as a nutrient source.

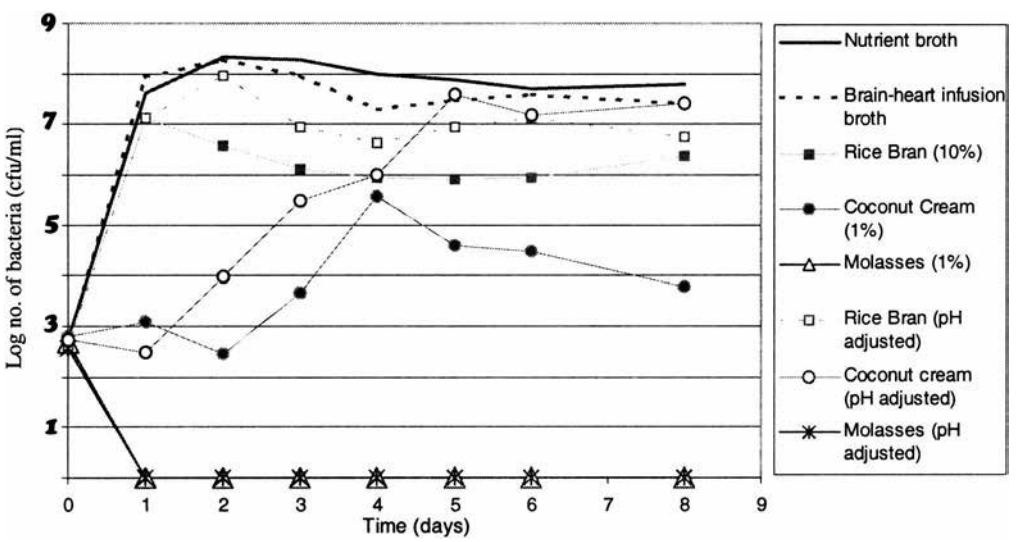

Figure 6. Growth of probiotic strain C1 in various microbiological grade and unrefined media using agricultural by-products

It is clear from this result that probiotic strain C1 can be mass produced using a cheap nutrient source like 10% rice bran extract as long as the pH of the medium is kept within neutral range.

FUTURE PLANS

The above results show promise for bacterial strain C1 as a probiotic. However, a lot more need to be studied regarding its application in crustacean hatcheries. More basic studies to explain the exact mode of action of bacterial probiotics need to be done. A major task ahead is to determine the stability of the microbial environment after C1 application and to develop rearing protocols that will guarantee the attainment of crab and shrimp survival values that are significantly different from those without probiotic application. In addition to improving the survival of hatchery-reared crustacean larvae, more studies for C1 application need to be done to ensure that the associated probiotic bacteria will remain in the animals during grow-out culture.

Although strain C1 lends itself to mass production using a cheap medium of 10% rice bran extract, an important quality control criteria has to be developed to guarantee that no genetic alteration leading to loss of efficacy will occur.

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Integration of Finfish in Shrimp (*Penaeus monodon*) Culture: An Effective Disease Prevention Strategy

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ABSTRACT

A farm trial on integration of finfish (*i.e.*, tilapia) in shrimp (*Penaeus monodon*) culture was conducted in Negros Occidental, Philippines to prevent luminous vibriosis in shrimp. The farm engaged in shrimp monoculture from 1987 to 1995. However, the prevailing luminous vibriosis outbreaks that started in 1994 prompted the farm operator to shift to tilapia culture in 1995-1996. The farm resumed shrimp operations in 1996 but by this time tilapia had already been integrated in the culture system. This paper reports on the results of the trial for 1999 using three ponds (ponds 7, 9, 29). These ponds had previously been used for tilapia culture for two years. During shrimp culture, they drew water from reservoirs stocked with tilapia and within the shrimp ponds tilapia are also stocked inside cages. This technology integrates crop rotation, biological pre-treatment and polyculture into one system. During the culture period the chemical and bacteriological quality of soil, water and shrimp were monitored. Water quality parameters were within normal ranges for shrimp culture. Luminous bacterial counts in water and shrimp were consistently below 10 colony forming units (cfu)/ml and 10^3 cfu/hepatopancreas (hp), respectively. These levels are below threshold levels associated with luminous vibriosis outbreaks. With a stocking density of 19.43 shrimp postlarvae (PL)/m^2, pond 7 yielded 2,605 kg shrimp/ha with an estimated survival of 35.65% after 109 days of culture (DOC). With a stocking density of 18.69 PL/m^2, pond 9 yielded 5,472 kg shrimp/ha with survival of 100% after 148 DOC. With a stocking density of 19.33 PL/m^2, Pond 29 yielded 5,702 kg shrimp/ha with survival of 82.66% after 151 DOC. The relatively low production in pond 7 can be attributed to the inferior quality of the batch of stocked shrimp PL that already had a low survival of 50% at DOC 30. Comparing the production performance from this present trial with that of this and other farms before the 1994 outbreaks, these good results cannot simply be attributed to chance despite of the lack of control in this farm trial. These results are consistent with the results of a previous trial of the same farm, the on-going verification trials in Negros Occidental, and the observations of many farmers in other parts of the country on the potential of shrimp-finfish integration in preventing luminous vibriosis in shrimp.

INTRODUCTION

Shrimp (*Penaeus monodon*) production in the Philippines peaked at 30,462 MT in 1991. Negros Occidental, the hub of intensive shrimp fanning in the Philippines, registered the highest production of 1,000 MT/month in the early 1990s. However, because of disease outbreaks (usually luminous vibriosis) production dropped to less than 100 MT/month in late 1994 and has not recovered since.

When the first outbreaks occurred in late 1994, the Bureau of Fisheries and Aquatic Resources (BFAR) immediately dispatched a technical team to investigate the problem. Throughout these past few years, numerous farm trials on the use of antibiotics and disinfectants (Baticados and Paclibare, 1992); bioaugmentation (Boyd and Gross, 1998); nutritional enrichment (Darachai *et al*., 1998); ozonation (Matsumura *et al*., 1998); immunostimulation (Horne *et al*., 1995); and ultraviolet (UV)-disinfection were either conducted or monitored by BFAR and the Negros Prawn Producers' Marketing Cooperative, Inc. (NPPMCI). However, none of these techniques proved consistently effective.

Luminous vibriosis in the Philippines is usually caused by *Vibrio harveyi* and high numbers of the bacterium in water has been associated with numerous disease outbreaks (Lavilla-Pitogo and dela Peña, 1998). In a 1994-1995 pond monitoring done by Lavilla-Pitogo (1998), they found that the onset of mortalities was always preceded by an exposure of the shrimp postlarvae (PL) to a high luminous bacterial population (10^2 to above 10^4 colony forming units (cfu)/ml) for three or more days.

In 1995-1996, laboratory analysts of NPPMCI monitored the water and soil quality of a semi-intensive shrimp farm in E.B. Magalona, Negros Occidental. In this set-up, polluted water (contaminated by domestic and agro-industrial pollutants) was pre-treated in a reservoir where tilapia was being cultured. The luminous bacterial count (LBC) of the water usually decreased from 10^2-10^3 to 10^1-10^2 cfu/ml (LBC in reservoir). A portion of this reservoir water was then transferred to the shrimp pond and the addition of this water caused the temporary decrease in LBC in the shrimp pond from the usual l0^1-l0^2 to >10^1-10^1. After a few days, the LBC in the shrimp pond would return to the usual 10^1-10^2 cfu/ml. This finding led us to try combining biological pre-treatment and polyculture. We hypothesized that tilapia culture can potentially decrease LBC but this potential can only be maintained if tilapia are also cultured simultaneously in the same pond containing the shrimp. Trials were therefore conducted in that farm and in the NPPMCI Demonstration Farm, Bago City, Negros Occidental. These early attempts on integrating tilapia in semi-intensive shrimp culture did not produce satisfactory results. However, the many insights learned from those trials were applied in two farms in San Enrique and Pulupandan, Negros Occidental.

Since 1996, these two farms have been integrating tilapia in their shrimp culture operations as an alternate species in crop rotation, as a biological pre-treatment agent in reservoir, and as an additional species in polyculture. Crop rotation is the culture of one organism after another in a same area. It is a type of "sanitation" practice which reduces initial inoculum of pathogen to a sufficiently low level so that the normal development of pathogen population will not reach a high level enough to cause appreciable yield loss, provided there is no unusual influx of pathogens (Berger, 1977). Biological pre-treatment is the use of an organism that causes the improvement of

quality of incoming water. Polyculture is the simultaneous cultivation of two or more organisms in an area and may improve soil and water quality during the culture period.

This paper presents the results of these trials on one of these two farms in Negros Occidental. The trials on the other farm are still in progress.

MATERIALS AND METHODS

Farm

The farm is located in San Enrique, Negros Occidental, four kilometers from the sea. It has 32 ponds with a total area of 43 hectares (ha). The farm started shrimp culture operation in 1982 and experienced luminous vibriosis outbreaks in 1994. The farm started to culture tilapia in 1996 because of the drop in market price of shrimp and the unavailability of technology to address the luminous vibriosis problem.

Water source

The farm draws brackish water (8-20 ppt) from the adjacent Bagonawan River. The luminous bacterial count of the river water ranges from 10^1 to 10^2 cfu/ml. The water goes to a sedimentation pond (around 4 ha) before it is transferred to the reservoirs stocked with tilapia. Although the use of sedimentation pond is not essential, the fanner decided to use one of his unutilized ponds for this purpose to receive the relatively turbid water from the river before transferring it to the reservoirs.

Reservoirs (Tilapia culture ponds)

The farm maintains more than one reservoir for every culture pond to help ensure a ready supply of good quality water at all times. (Ponds 2, 8, 21, 28 and 31, having areas of 1.24, 1.00, 0.66, 0.52, and 0.43 ha, respectively, served as reservoirs).

Table 1 shows a brief history of these ponds.

Table 1. Culture operations of ponds 2, 8, 21, 28 and 31 from 1992 to 1999

Year	Pond 2	Pond 8	Pond 21	Pond 28	Pond 31
1992	shrimp culture	shrimp culture	shrimp culture	shrimp culture	shrimp culture
1993	shrimp culture	shrimp culture	shrimp culture	shrimp culture	shrimp culture
1994	shrimp culture	shrimp culture	shrimp culture	shrimp culture	shrimp culture
1995	shrimp culture	shrimp culture	shrimp culture	shrimp culture	shrimp culture
1996	shrimp culture	tilapia culture	shrimp culture	tilapia culture	shrimp culture
1997	tilapia culture	-	tilapia culture	-	tilapia culture
1998	tilapia culture	-	tilapia culture	-	tilapia culture
1999	tilapia culture	tilapia culture	tilapia culture	tilapia culture	tilapia culture

The reservoirs were prepared to favor the growth of natural food. They were stocked with 3-4 saline tolerant tilapia/m^2. The farm produces its so-called "Jewel Tilapia" which is a hybrid of *Oreochromis mossambicus* and *T. hornorum* imported from the USA. Supplemental feeding with commercial feed started after 30 days of culture (DOC). Aeration started at DOC 90 when the biomass reached around 3 tons/ha. During nighttime, the aerators were operated for 12 h, from 6 PM to 6 AM. During daytime, aerators were occasionally operated (*e.g.*, before water was transferred to shrimp ponds and depending on weather conditions).

A tilapia biomass of 3,000-3,500 kg/ha was maintained in reservoirs. It is at this biomass that the luminous bacterial count becomes undetectable and the water quality is stable.

Shrimp-tilapia culture ponds

For the trial being described, three ponds were used - ponds 7, 9 and 29 having areas of 1.0007 ha, 0.931 ha, 0.5276 ha, respectively. The pond preparation followed the industry practice.

For the design and placement of tilapia cages, four cages with dimensions of 10 m x 10 m x 10 m were placed around and 0.5 m away from the pond center. Each cage had two layers of nets: a fine-meshed net and a large-meshed net. To enhance water circulation within and outside the cage, the fine-meshed net was removed when the shrimp were big enough (average body weight, ABW, 5 g) not to enter the tilapia cages. The bottom of the cages had a distance of 0.3 m from the pond bottom.

Tilapia weighing 100 g each was stocked in the cages as soon as the ponds were filled with water from the reservoir. A total tilapia biomass of 400 kg/ha was stocked in four cages in each of the three ponds.

Shrimp PLs (PL 16-18; 16-18 days after molting to postlarval stage) were stocked in the pond at around 18-19 PL/m^2. Since the shrimp pond water came from the reservoirs (stocked with tilapia) and the chemical and microbial quality of the water was determined to be acceptable (*e.g.*, Secchi disc visibility, 30-60 cm, pH, 7.5-8.5, luminous bacterial count, less < than 10^1 cfu/ml), shrimp PLs were stocked 5-7 days after water filling.

Table 2 shows a brief history of these ponds. Ponds 7 and 9 were devoted to shrimp-milkfish (*Chanos chanos*) polyculture from 1982 to 1987. Pond 29 started shrimp culture operation in 1988.

Table 2. Culture operations of ponds 7, 9 and 29 from 1987 to 1999

Year	Pond 7	Pond 9	Pond 29
1987	shrimp culture	shrimp culture	-
1988	shrimp culture	shrimp culture	shrimp culture
1989	shrimp culture	shrimp culture	shrimp culture
1990	shrimp culture	shrimp culture	shrimp culture
1991	shrimp culture	shrimp culture	shrimp culture
1992	shrimp culture	shrimp culture	shrimp culture
1993	shrimp culture	shrimp culture	shrimp culture
1994	shrimp culture	shrimp culture	shrimp culture
1995	shrimp culture	shrimp culture	shrimp culture
1996	tilapia culture	tilapia culture	shrimp culture
1997	tilapia culture	tilapia culture	tilapia culture
1998	-	integrated shrimp-tilapia culture	tilapia culture
1999	integrated shrimp-tilapia culture	integrated shrimp-tilapia culture	integrated shrimp-tilapia culture

Soil and water quality monitoring

Before pond preparation and after harvest, pond soil was analyzed for organic matter (OM) content and pH using standard methods of soil analyses (Boyd and Tucker, 1992). Pond water quality was monitored closely. Dissolved oxygen (DO) was measured using DO meter four times a day at 4:00 P.M., 10:00 P.M., 2:00 A.M., and 4:00 A.M.. Temperature, salinity, pH, secchi disc visibility, depth, color and weather were recorded twice a day at 6:00 A.M. and 3:00 P.M.

Unionized ammonia, nitrite, alkalinity, and phytoplankton count were determined occasionally using standard methods (Strickland and Parsons 1972; Boyd and Tucker, 1992). Although water quality parameters were determined daily throughout the culture periods for both ponds 9 and 29, for pond 7 DO and temperature were determined only on DOC 17-57 and pH, salinity, transparency and depth were determined only on DOC 1-77.

Quantitative bacteriology

Quantitative bacterial counts on pond water and shrimp were made twice a week using standard methods (Lavilla-Pitogo *et al*., 1998; Leaño *et al*., 1998). For water samples, ten-fold serial dilutions were made and 0.1 ml aliquots of the dilutions were plated in duplicate on two culture media: nutrient agar (NA) and thiosulphate citrate bile-salt sucrose (TCBS) agar. For shrimp samples, five random samples were dissected aseptically and the hepatopancreas tissues (hp) removed, homogenized and suspended in sterile seawater (SSW) at one hp/ml SSW. Aliquots (0.1 ml) of the serially diluted homogenates were also spread on NA and TCBS plates. Total plate count (TPC), LBC, and presumptive *Vibrio* count (PVC) were determined after 18-24 h incubation at room temperature. Quantitative bacteriology of reservoir, source water and pond soil was also conducted occasionally.

Water management

Replenishment water was drawn from the reservoirs. The decision to replenish water was based on laboratory analysis and pond observation. Starting on DOC 17, 10-15% of the water in the shrimp-tilapia culture ponds was replaced with water from the reservoirs during water exchange.

Feeding management

The feeding of shrimp followed industry practice using commercial pellets and supplemental food (*e.g*., golden snail *Pomacea canaliculata*). The tilapia was also fed with commercial pellets.

Shrimp growth and health monitoring

Shrimp samples (n=5) were taken randomly and weighed every five days starting at DOC 30 until harvest time. Gross inspection of shrimp for signs of disease was done during this sampling period.

RESULTS

Production

Ponds 9 and 29 had good shrimp production of more than 5,000 kg/ha and more than 80% survival (Table 3). Pond 7 had a lower production which is explained below in the Discussion.

Table 3. Shrimp production in ponds 7, 9 and 29

Pond number	Stocking density (PL/m^2)	DOC at harvest (days)	Total production (kg/ha)	ABW (g)	Survival (%)	FCR
7	19.43	109	2,605	37.61	35.7	1.17
9	18.69	148	5,472	29.15	100	1.70
29	19.33	151	5,702	35.69	82.66	1.69

PL – postlarvae
DOC – days of culture
ABW – average body weight
FCR – feed conversion ratio

In 1998, pond 9 was also used for integrated shrimp-tilapia culture. With a stocking density of 18 PL/m^2, shrimp production was 4,385 kg/ha after DOC 108 with survival of 90%.

The tilapia production from ponds 7,9 and 29 are shown in Table 4. The tilapia production data from the reservoirs are not available because the intention is not to remove them from the reservoirs.

Table 4. Tilapia production performance of ponds 7, 9 and 29

Pond number	Stocking density (fish/m^2)	DOC at harvest (days)	Total production (kg)	ABW (g)	Survival (%)	FCR
7	10	101	1,250	313	100	1.77
9	10	148	1,500	375	100	1.70
29	10	151	1,200	300	100	1.69

DOC - days of culture
ABW - average body weight
FCR - feed conversion ratio

Soil and water quality

The OM and pH of the pond soil ranged from 1.75% to 2.5% and 6.9 and 7.4, respectively. Water quality parameters were within ideal ranges for shrimp culture (Table 5.) Dissolved oxygen, temperature and pH changed significantly during the day while salinity and transparency did not.

Table 6 shows the differences of water quality among ponds 7, 9 and 29. Dissolved oxygen and temperature were compared for DOC 17-57 because it is only during that period that data from the three ponds can be compared. For the other parameters, ponds were compared for the first 77 days.

From DOC 17-57, the DO and temperature of ponds 7,9 and 29 did not differ from each other. Likewise, pH was also not significantly different for the three ponds for the first 77 days of their culture.

Table 7 shows the correlation of the various water parameters with each other. Dissolved oxygen was directly related to salinity ($P < 0.05$) while depth was likewise directly related to temperature ($P < 0.05$) and salinity ($P < 0.001$).

Chlorella spp. was the most numerous species of phytoplankton present throughout the culture period (89.2%±9.5%, 86.6%±8.5%, 85.8%±7.4% for ponds 7, 9, and 29, respectively).

Table 5. Overall means and standard deviation (SD) of water quality parameters at different sampling times for ponds 7, 9 and 29

Pond Number	Dissolved oxygen (ppm)				Temperature (°C)		pH		Salinity (‰)		Transparency (cm)		Depth (cm)	
	4PM	10PM	2AM	4AM	6AM	3 PM	6AM	3 PM	6AM	3 PM	6AM	3 PM	6AM	3 PM
7	10.2 ± 1.9[a]	6.0 ± 1.1[b]	5.0 ± 1.0[c]	4.0 ±0.8[d]	29.3 ± 1.6[a]	31.5 ± 1.9[b]	7.6 ± 0.2[a]	8.1 ± 0.1[b]	11.3 ± 1.7[a]	11.2 ± 1.7[a]	29.1 ± 2.4[a]	28.8 ± 2.3[a]	91.2 ± 3.5[a]	91.4 ± 3.6[a]
9	10.9 ± 2.1[a]	5.9 ± 1.4[b]	4.6 ± 1.1[c]	3.8 ± 1.0[d]	29.2 ± 1.5[a]	31.4 ± 1.7[b]	7.7 ± 0.1[a]	8.1 ± 0.1[b]	11.8 ± 2.6[a]	11.8 ± 2.6[a]	25.1 ± 2.9[a]	24.9 ± 2.9[a]	94.9 ± 9.0[a]	98.2 ± 3.7[b]
29	10.0 ± 3.3[a]	5.8 ± 1.4[b]	4.7 ± 1.2[c]	4.0 ± 1.1[d]	29.2 ± 1.6[a]	31.2 ± 1.9[b]	7.7 ± 0.2[a]	8.1 ± 0.2[b]	11.7 ± 2.4[a]	11.7 ± 2.3[a]	25.6 ± 4.3[a]	25.2 ± 4.1[a]	95.4 ± 9.0[a]	99.0 ± 2.5[b]

Means of each water quality parameter within a row with different superscripts are significantly different ($P < 0.05$)

Table 6. Means and standard deviation (SD) of water quality parameters at days of culture (DOC) 17-57 for dissolved oxygen and temperature; and DOC 1-77 for pH, salinity, transparency and depth for ponds 7, 9, and 29

Pond number	Dissolved oxygen (ppm)	Temperature (C)	pH	Salinity (‰)	Transparency	Depth
7	6.3±2.7[a]	30.4±2.0[a]	7.9±0.3[a]	11.2 ± 1.7[a]	29.0±2.8[a]	91.3±3.5[a]
9	7.0±2.4[a]	30.9±1.5[a]	7.9±0.2[a]	12.9±2.9[b]	26.3±2.9[c]	96.6±5.5[b]
29	7.0±2.9[a]	31±1.5[a]	7.9±0.2[a]	13.0±2.4[b]	27.5±4.5[b]	97.0±5.3[b]

Means within a column with different superscripts are significantly different (P<0.05)

Table 7. Pearson coefficients of correlation* between means of water quality parameters in ponds 7,9 and 29. (DOC 17-57 for dissolved oxygen and temperature; DOC 1-77 for pH, salinity, transparency and depth)

	Dissolved oxygen (ppm)	Temperature (°C)	pH	Salinity (‰)	Transparency	Depth
Dissolved oxygen	x					
Temperature	0.987	x				
pH	0.950	0.988	x			
Salinity	0.998	0.995	0.968	x		
Transparency	-0.897	-0.814	-.716	-0.868	x	
Depth	0.995	0.997	0.976	0.999	0.999	x

* r>0.997, P<0.05; r>0.999, P<0.001

Quantitative bacteriology

Luminous bacterial counts were consistently lower than 10^1 cfu/ml and 10^3 cfu/hp in pond water and shrimp, respectively (Table 8). Table 9 compares the three ponds in terms of bacterial counts in water and shrimp during the first 109 DOC.

Although the three ponds varied greatly in terms of TPC and PVC, all of them consistently had LBC lower than 10^1 cfu/ml and 10^3 cfu/hp in pond water and shrimp, respectively, for the first 109 DOC. The Pearson correlation analysis of the above data revealed no relationship between the TPC of water and shrimp (r=-0.438) and PVC of water and shrimp (r =-0.807). The LBC of water and shrimp could not be compared because of their inexact values.

Table 8. Overall means for bacterial counts for water and shrimp in ponds 7, 9 and 29

Pond number	TPC, water (cfu/ml)	PVC, water (cfu/ml)	LBC, water (cfu/ml)	TPC, shrimp (cfu/hp)	PVC, shrimp (cfu/hp)	LBC, shrimp (cfu/hp)
7	2.9×10^4	2.2×10^3	$<10^1$	1.9×10^7	4.0×10^5	$<10^3$
9	1.4×10^4	1.4×10^3	$<10^1$	1.8×10^7	2.4×10^5	$<10^3$
29	1.4×10^4	1.4×10^3	$<10^1$	1.8×10^7	2.4×10^5	$<10^3$

Means within a column with different superscripts are significantly different ($P<0.05$)
TPC - total plate count
PVC - presumptive *Vibrio* count
LBC - luminous bacterial count
cfu - colony forming unit
hp - hepatopancreas

Table 9. Means of bacterial counts for water and shrimp in ponds 7, 9 and 29 for the first 109 days of culture (DOC)

Pond number	TPC, water (cfu/ml)	PVC, water (cfu/ml)	LBC, water (cfu/ml)	TPC, shrimp (cfu/hp)	PVC, shrimp (cfu/hp)	LBC, shrimp (cfu/hp)
7	2.9×10^{4b}	2.2×10^{3b}	$<10^1$	1.9×10^{7b}	4.0×10^{5c}	$<10^3$
9	1.5×10^{4c}	1×10^{3a}	$<10^1$	1.7×10^{7a}	3.2×10^{5b}	$<10^3$
29	1.0×10^{6a}	1.5×10^{7c}	$<10^1$	1.7×10^{7a}	2.4×10^{5a}	$<10^3$

Means within a column with different superscripts are significantly different ($P <0.05$)
TPC - total plate count
PVC - presumptive *Vibrio* count
LBC - luminous bacterial count
cfu - colony forming unit
hp - hepatopancreas

Figures 1 to 6 show the changes of bacterial counts in water and shrimp in the three ponds. The LBC was consistently lower than PVC and TPC. Except for one time, the LBCs of water and shrimp never exceeded 10 cfu/ml and 1,000 cfu/hp, respectively.

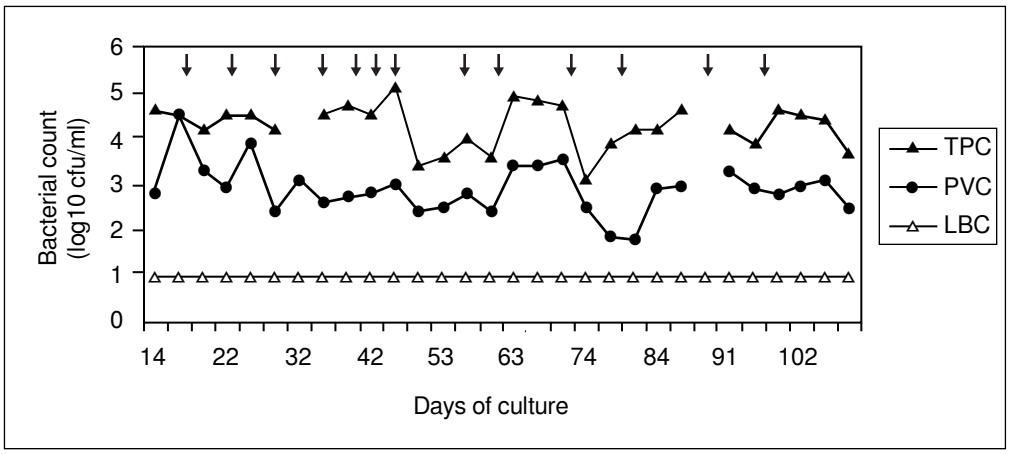

Figure 1. Bacterial count in water of pond 7. The dates when water from tilapia reservoir was added are indicated by an arrow. TPC - total plate count; PVC presumptive - *Vibrio* count; LBC - luminous bacterial count; cfu - colony forming unit

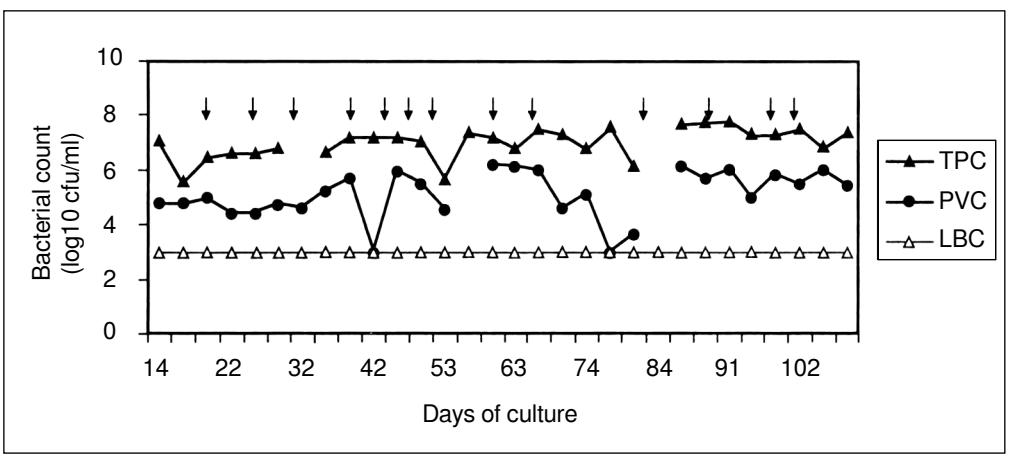

Figure 2. Bacterial count in shrimp of pond 7. The dates when water from tilapia reservoir was added are indicated by an arrow. TPC - total plate count; PVC - presumptive *Vibrio* count; LBC - luminous bacterial count; cfu - colony forming unit; hp - hepatopancreas

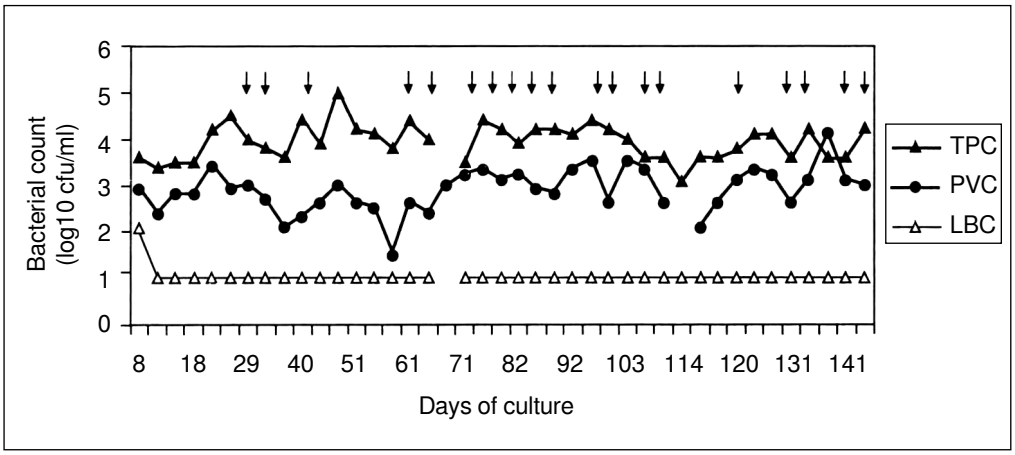

Figure 3. Bacterial count in water of pond 9. The dates when water from tilapia reservoir was added are indicated by an arrow. TPC - total plate count; PVC - presumptive *Vibrio* count; LBC - luminous bacterial count; cfu - colony forming unit

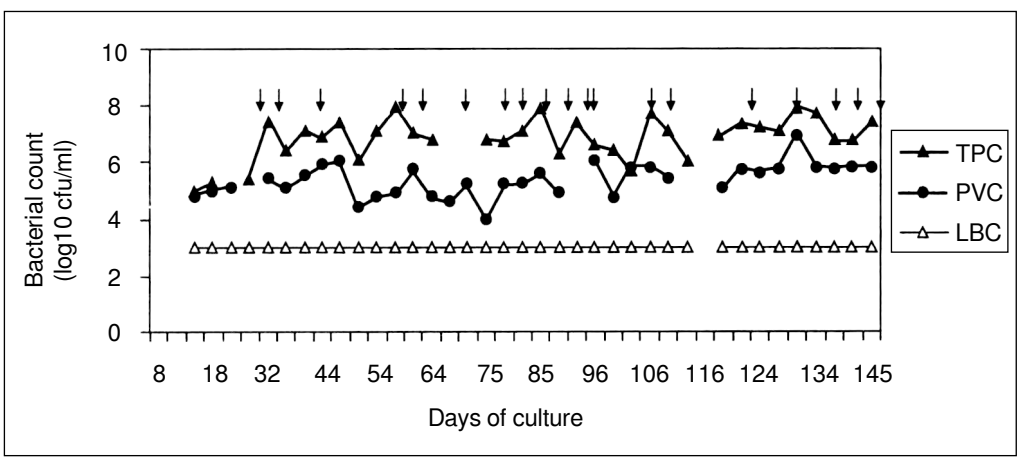

Figure 4. Bacterial count in shrimp of pond 9. The dates when water from tilapia reservoir was added are indicated by an arrow. TPC - total plate count; PVC - presumptive *Vibrio* count; LBC - luminous bacterial count; cfu - colony forming unit; hp - hepatopancreas

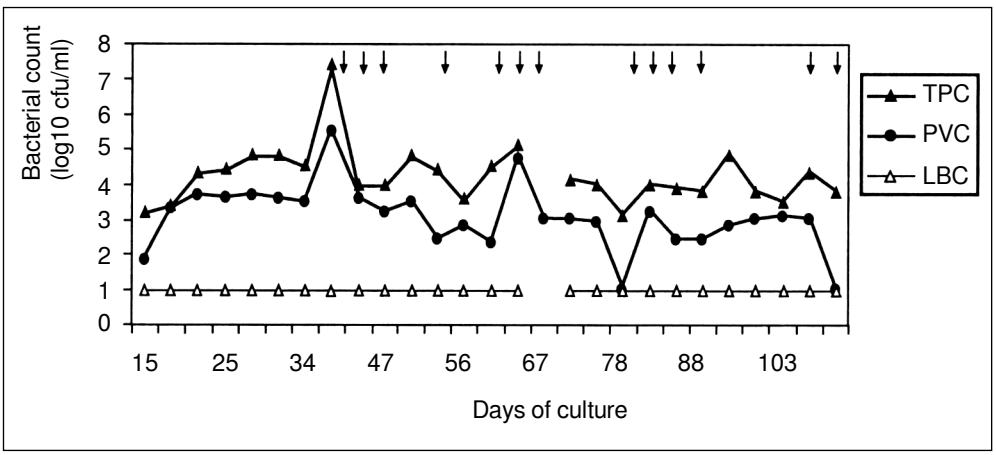

Figure 5. Bacterial count in water of pond 29. The dates when water from tilapia reservoir was added are indicated by an arrow. TPC - total plate count; PVC - presumptive *Vibrio* count; LBC - luminous bacterial count; cfu - colony forming unit

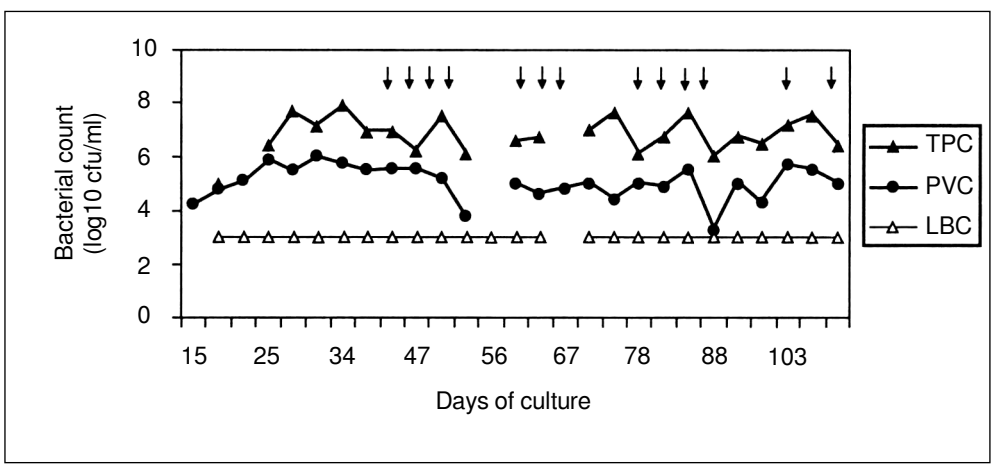

Figure 6. Bacterial count in shrimp of pond 29. The dates when water from tilapia reservoir was added are indicated by an arrow. TPC - total plate count; PVC - presumptive vibrio count; LBC - luminous bacterial count; cfu - colony forming unit; hp - hepatopancreas

DISCUSSION

Shrimp production

Although there was no control pond for this farm trial, the good production performance of Ponds 9 and 29 cannot simply be attributed to chance.

Table 10 shows progressive decline in shrimp survival of various ponds in FSD Farm from 1992 to 1996.

Table 10. Survival in shrimp monoculture of FSD Farm from 1992 to 1996

Year	Pond 7	Pond 9	Pond 29	Pond 2	Pond 8	Pond 21	Pond 28	Pond 31
1992	89.7	73.6	55.3	23.2	35.4	86.5	79.4	98.3
1993	75.3	75.6	85.3	78.8	74.6	55.7	59.4	86.4
1994	?	42	?	?	26	24.5	?	35
1995	40.48	28	21.2	16	14	11	27.6	11
1996	*	-	*	*	-	40	-	15

* Aborted operation
− No shrimp culture operation
? Missing data

Luminous vibriosis has been prevalent not only in Negros Occidental but also in other areas (*e.g.* the nearby province of Iloilo). A certain farm in Iloilo, for instance, had declining yearly survival rates of 86.6%, 63% and 29.3% from 1992 to 1994 (Lavilla-Pitogo *et al.*, 1998). Table 11 shows the decline in the production of export quality shrimp in the Philippines starting in 1995. The sharp decline in 1995 apparently reflects the first outbreaks of luminous vibriosis throughout the country in 1994.

Table 11. Philippine shrimp export from 1987 to 1997

Year	Quantity (MT)
1987	14,935
1988	24,288
1989	26,052
1990	24,146
1991	31,156
1992	23,003
1993	22,206
1994	21,518
1995	12,095
1996	13,514
1997	10,532

In view of the many unsuccessful attempts to control luminous vibriosis in other farms for the past few years, the declining shrimp survival in these farms suggests that only an effective control strategy can reverse the trend. The survival in ponds 9 and 29 in 1995 was 28 and 21%, respectively. With this present intervention, the survival in both ponds increased tremendously to 100% and 82.66%.

The relatively low shrimp production in pond 7 can be attributed to the inferior quality of stocked postlarvae. At DOC 30, the survival was already estimated at a low 50% and appeared to be unrelated to *V. harveyi.* In all the three ponds, for the first 30 DOC, the LBC for the shrimp was < 10^3 cfu/hp and <10^1 cfu/ml for water. Likewise, water quality was similar for all three ponds.

The on-going verification trial in another farm (Pulupandan, Negros Occidental) is also showing promising results. The results of this present trial and other on-going trials are consistent with the observation of many shrimp farmers throughout the country that crop rotation and shrimp-finfish polyculture is a promising technique for avoiding shrimp losses.

Finfish-based biological control

The first outbreaks of luminous vibriosis in grow-out ponds in the Philippines occurred in 1994. Many of these luminous vibriosis outbreaks have been associated with phytoplankton die-off (Paclibare, 1998). Another major risk factor is the high number of luminous bacteria (10^2 to above 10^4 cfu/ml) in pond water especially during the first 45 days of culture (Lavilla-Pitogo *et al*., 1998).

In a shrimp pond system, the most likely sources of luminous bacteria are the soil and incoming water (Karunasagar *et al*., 1996a; Lavilla-Pitogo *et al*., 1998). As soon as the pond is filled with water, the luminous bacterial population tends to increase, probably because of the organic matter remaining after pond preparation (Lavilla-Pitogo *et al*., 1998; Smith, 1998). During the culture period, organic matter (uneaten feed, shrimp faeces, exuviae, and plankton debris) accumulated. It is expected therefore that the luminous bacteria population would also increase.

The luminous bacterial population in soil and water can be reduced by either chemical or biological approaches. However, it is difficult to reduce the luminous bacterial population by disinfectants and antibiotics because the bacteria are protected in biofilms. Bacterial biofilms are composed of populations or communities of microorganisms adhering to environmental surfaces. These microorganisms are usually encased in an extracellular polysaccharide that they themselves synthesize. Biofilms may be found on essentially any environmental surface in which sufficient moisture is present. Biofilms are notably resistant to drying and disinfection. The role of biofilms in the development of bacterial diseases in shrimp ponds has not been investigated yet. However, the findings of Karunasagar *et al.* (1994; 1996b) may have some relevance to shrimp ponds. Karunasagar *et al.* (1994) reported mass mortality of *P. monodon* larvae due to an antibiotic-resistant *V. harveyi* infection and suggested that antibiotic-resistant, virulent strains of *V. harveyi* were colonizing larval tanks. In their follow-up study (Karunasagar *et al*., 1996b), they observed that *V. harveyi* can form biofilms on all three substrates they tested: cement slabs, high density polyethylene plastic and steel surfaces. Furthermore, the bacteria in biofilm were found to be more resistant to chlorine disinfection than their planktonic counterparts. In shrimp ponds, Karunasagar *et al.* (1996a) found that *V. harveyi* can survive even in sediments that are treated

with high doses of disinfectants. The addition of unspecified type of lime at 100 ppm to microcosms containing pond soil and water affected only *V. harveyi* counts in the soil marginally. The addition of chlorine at 10 ppm also led to a slight reduction of *V. harveyi* counts followed by an increase. When experiments were conducted on bacterial populations suspended in seawater, *V. harveyi* was found to be completely eliminated after 30 min exposure to 10 ppm chlorine.

Because of the difficulty in reducing the concentration of pathogenic bacteria in shrimp ponds by conventional chemical disinfection, other effective means such as biological control have been explored. Biological control may be divided into two approaches: a) the addition into the environment of beneficial microorganisms that serve as antagonists of the target pathogens and b) the manipulation of the environment in such a way that the proliferation of beneficial microorganisms is favored. Examples of these approaches are bioaugmentation (Boyd and Gross, 1998) and crop rotation, respectively. Experiences on bioaugmentation in shrimp culture indicate conflicting results. For instance, many of the commercial bioaugmentation products that were claimed by their producers to be effective did not perform well in Negros Occidental. Assuming bioaugmentation can reduce pathogenic bacteria in shrimp ponds, the method may still not be cost-effective because high amounts of the costly bioaugmentation products must be added to the ponds frequently. The second approach, *i.e.*, crop rotation is increasingly being recognized as an effective disease control strategy in shrimp culture similar to agriculture.

In the field of agriculture in Peru, for instance, a mandatory seven-year rotation for potatoes was established before the arrival of the Spaniards. It is now known that this practice was used to control potato cyst-nematodes (Sieczka, 1989). Kommendahl and Todd (1991) list approximately 64 fungal, 19 nematodes, 1 viral and 16 bacterial disease in plants in which crop rotation was found to be an effective control practice.

Crop rotation as applied in shrimp culture addresses problems due to luminous bacteria (and also possibly other pathogens) in the pond soil previously used for shrimp culture. The continuous monoculture of shrimp over the past few years may have caused the increase in luminous bacteria in the culture environments. Although many of the farms employ thorough pond preparation techniques, these bacteria may be carried over into succeeding cultures as they may be protected by bacterial biofilms. When a different kind of aquatic organism is reared in the pond previously used for shrimp culture, a different microenvironment is created. This change in the microenvironment may cause a change in the microflora of the pond soil. It has been observed that the greater the phylogenetic differences between the culture organisms used in crop rotation, the better the sanitary effect (Francis and Clegg, 1990). Since shrimp and finfish (*e.g.*, tilapia) belong to different orders within the animal kingdom, they are considered good candidates for crop rotation.

The potential of crop rotation in shrimp culture is also now being recognized in other countries. In Indonesia, Akiyama and Anggawati (1998) mentioned about an interesting case of a problematic shrimp pond which may lend support to the value of crop rotation. In the first semester of 1994, this pond had emergency harvest. During the succeeding three cycles, the operation had to be aborted within 40 days. In 1996, this pond was used instead as a tilapia broodstock pond. In the first semester of 1997, this pond was used for intensive monoculture of shrimp. During this cycle the production level was 5.2 MT/ha of 19g shrimp at 59% survival with FCR of 1.9. Apparently, the culture of tilapia may have “rejuvenated the pond.” Other countries like Bangladesh (Hossain, 1998) and Thailand (P. Chanratchakool, pers. com.) are also having

good experiences on shrimp-finfish crop rotation.

Since crop rotation can only be effective if there is no influx of luminous bacteria in the pond system, other sources of the bacteria should also be addressed. Biological pre-treatment using finfish and polyculture with finfish can be used to reduce luminous bacteria from incoming water and rearing water, respectively. In this farm trial, the LBC in all three ponds (ponds 7,9 and 29) were maintained below 10 cfu/ml. This level is much lower than the levels previously associated with luminous vibriosis outbreaks (10^2 to above 10^4 cfu/ml; Lavilla-Pitogo *et al.*, 1998). During the previous trial of the farm (using pond 8 as a reservoir stocked with tilapia and pond 9 as shrimp-tilapia pond), LBC would reach non-detectable level once the biomass of tilapia at the reservoirs reached 3,500 kg/ha. That biomass of tilapia was therefore maintained at the reservoirs during this present trial. Hence, maintaining low LBC at the shrimp-tilapia ponds which drew water from those reservoirs. The presence of tilapia in cages within the shrimp pond (at around 1,300 kg/ha biomass) may have also helped maintain that low LBC.

Aside from lowering LBC, tilapia culture may also have helped improve soil and water quality during this trial. In a trial on polyculture of shrimp and tilapia in Ecuador, Akiyama and Anggawati (1998) observed that water pH was maintained in the optimum range of 7.7 to 8.3 in polyculture ponds in contrast to the control ponds pH which ranged from pH 7.5 to 8.5. In addition, phytoplankton bloom in the polyculture ponds was more stable than that in the control ponds. It was also observed that upon harvest, the polyculture ponds did not smell as bad as the control ponds. The ecological role of tilapia in an intensive shrimp (*P. chinensis*)-tilapia culture was discussed by Ji-Qiao *et al.* (1998). Selective feeding of tilapia on large plankton, particularly zooplankton, results in a decrease in predatory pressure on small phytoplankton. These small phytoplankton have high productivity and fully utilize nutrients in the water due to their large absorptive surface area and low precipitating index. This may help explain the dominance of *Chlorella* spp. in the present trial. Furthermore, the disturbance by tilapia while swimming around enhances water movement and nutrient cycle. Nitrogen and phosphorus excreted by tilapia function as slow and even fertilization which helps maintain an optimal and constant biomass of phytoplankton. Tilapia has also a positive effect on dissolved oxygen. Dissolved oxygen level reached its peak and was significantly higher in enclosures with tilapia than in control enclosures (without tilapia) when the tilapia biomass was 300 kg/ha.

The term “finfish-based biological control” is hereby coined to describe the above approach. This approach uses finfish in crop rotation, biological pre-treatment and polyculture to manipulate the pond environment in such a way that beneficial microorganisms are favored to proliferate.

Research Needs

The present trial is among the first reported attempts to integrate finfish in shrimp culture as a disease prevention strategy. Because of the promising results obtained in this trial, refinements of the technology should be further investigated. Technology verification trials are now being conducted under various farm conditions in Negros Occidental. Controlled experiments to determine the effects of biomass and species of finfish on physico-chemical and microbiological quality of soil and water in shrimp culture are also worth pursuing. The information from these experiments will be useful in refining the technology.

As observed in many trials in Negros Occidental, there is a dominance of *Chlorella* spp. in brackishwater tilapia culture. Some hypothesize that *Chlorella* may be inhibiting luminous bacteria in nature. Direkbusarakom *et al.* (1997) found that extracellular products of *Chlorella* have antibiotic activity against shrimp pathogens *V. harveyi, V. parahaemolyticus* and *V. penaeicida.* However, one should bear in mind that the ability to produce an inhibitory material does not necessarily mean that it plays an ecological role (Sieburth, 1968). An excellent example is the autoinhibitor chlorellin from *Chlorella vulgaris.* Scutts (1964), cited by Sieburth, 1968, stated that "inhibitor production is not a general phenomenon with *Chlorella* and only occurs under certain conditions." The demonstration of ecologically sufficient concentrations of specific inhibitors under natural conditions is essential to the proof of their importance in ecology (Sieburth, 1968). As discussed above, *Chlorella* (and other phytoplankton) may be beneficial in improving water quality and may help reduce luminous bacteria indirectly but not directly as in the case of antibiosis. This hypothesis needs to be tested.

Tilapia culture may change the microenvironment in such a way that it favors the proliferation of microorganisms other than the luminous vibrios. Among these microorganisms may include the resident antagonists of luminous vibrios. Resident antagonists are biological agents present in the local environment which have the potential to interfere in the life processes of target pathogens. Other species of bacteria such as *V. alginolyticus* and *Bacillus* sp. have been shown to inhibit growth and reproduction of *V. harveyi* and its closely related species *V. parahaemolyticus* (Garriques and Arevalo, 1995; Rengpipat *et al*., 1998). The presence and activity of these resident antagonists in tilapia culture is therefore worth studying to further provide scientific basis on the finfish-based biological control.

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Vibrio harveyi and the 'green water culture' of *Penaeus monodon*

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ABSTRACT

The "greenwater culture" of the tiger shrimp, *Penaeus monodon*, is an innovative culture technique for the grow-out rearing of shrimps. This culture method involves the use of rearing water of tilapia for the rearing of tiger shrimp in grow-out ponds and or the polyculture of shrimp with tilapia. This culture technique was reported to prevent disease outbreaks attributed to luminescent *Vibrio.* To understand the possible mechanisms of luminous *Vibrio* control in the green water culture system several studies were conducted. This review summarizes the highlights obtained so far from these studies consisting of a) effect of rearing waters from tilapia culture and shrimp cultured with tilapia on *Vibrio harveyi*; b) estimation and preliminary identification of cultivable bacteria, fungi and phytoplankton flora associated with the "green water culture" system and c) detection of anti-*Vibrio harveyi* metabolites from bacteria, yeast, filamentous fungi and phytoplankton indigenous to the "green water culture" system.

INTRODUCTION

The giant tiger shrimp, *Penaeus monodon*, comprise 56% of total shrimp production in the world (Fig. 1). As a significant dollar earner for exported shrimp in the Philippines, its production significantly increased through the years until 1995 (Fig.2). By 1996, shrimp production in the country drastically declined. These severe losses in grow-out cultured shrimps were attributed to outbreaks of luminous vibriosis caused by *Vibrio harveyi* (Lavilla-Pitogo *et al*., 1998a; Leaño *et al*., 1998, Lio-Po, 1998; de la Peña *et al*., 2001). Similarly, luminous vibriosis in *P. monodon* juveniles was also reported in Thailand (Jiravanichpaisal, *et al*., 1994).

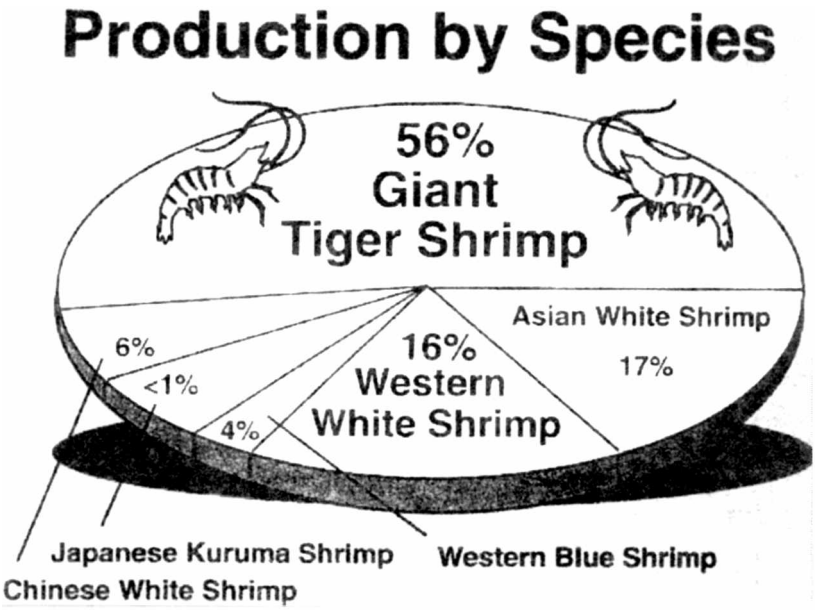

Figure 1. World production of shrimps by species (FAO, 2000)

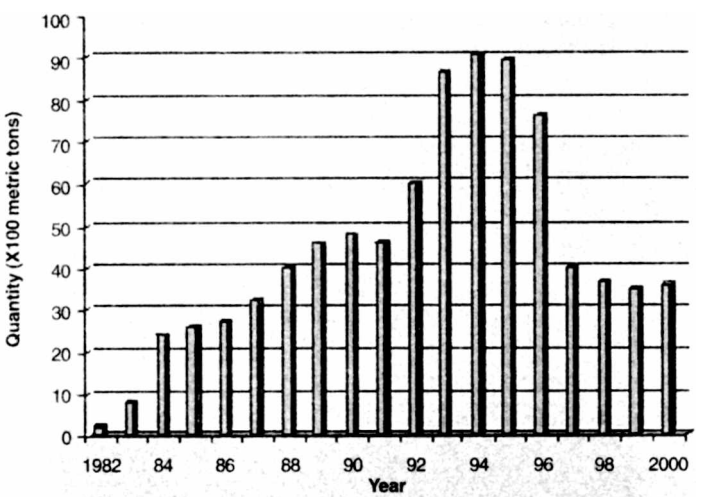

Figure 2. Shrimp production in the Philippines, 1982-2000 (BAS, 1983~2001)

To prevent the multiplication of luminous *Vibrio* in the shrimp grow-out ponds and to improve survival and production, innovations in shrimp culture techniques were developed (NPPMCI, 2000). Among these is the "green water culture technique" also known as "Tilapia integration to shrimp culture technique" (TIPS) for the grow-out culture of the tiger shrimp, *P. monodon*, initiated in 1999. The application of this rearing technique was reported to prevent the increase of *V. harveyi* in the ponds thereby preventing the onset of *V. harveyi* infections in the cultured shrimps (Fig. 3) (Platon, 1997; Corre *et al*. 2000). Briefly, this method utilizes water from tilapia culture ponds as the major source of rearing water for shrimp culture. In addition, tilapia may be stocked in cages in polyculture with the shrimp (Fig. 4).

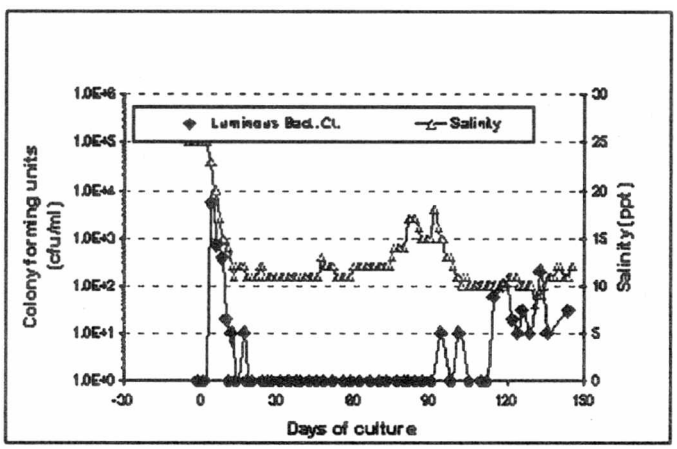

Figure 3. Luminous bacterial counts vs. salinity in a grow-out pond with *Penaeus monodon* applying the "greenwater culture" system (NPPMCI, 2000)

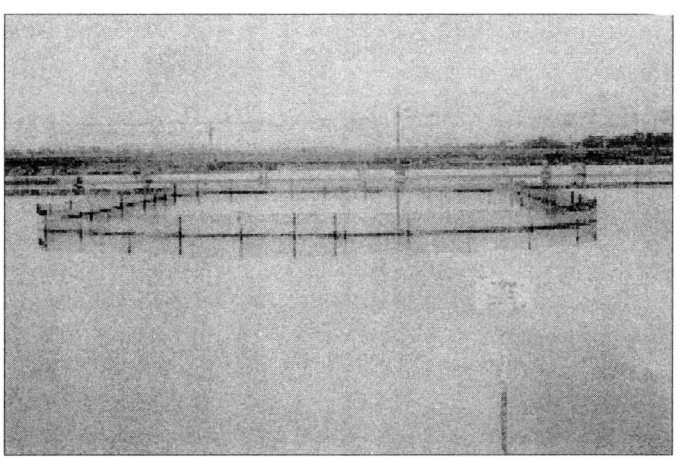

Figure 4. The 'green water culture" system in the grow-out culture of *Penaeus monodon* with Tilapia at the central cage

Because of the successful production runs attributed to the 'green water culture" system for the grow-out culture of *P. monodon*, this culture technique has gradually gained acceptance among shrimp farmers. However, the basis for its success needs to be investigated scientifically. This report summarizes the results of studies on the microbial flora associated with the "green water culture system" conducted at SEAFDEC-AQD namely: a) effect of the green water culture rearing waters on *V. harveyi*; b) estimation and preliminary identification of the cultivable bacterial, fungal and phytoplankton flora associated with the green water culture system; and c) detection of anti- *V. harveyi* metabolites from these microbial flora.

VIBRIO HARVEYI AND SHRIMP/TILAPIA CULTURE WATERS

Pure cultures of *V. harveyi* were exposed to rearing waters of shrimp grow-out culture, shrimp with tilapia culture, tilapia culture and their respective seawater sources in triplicates. Results did not show a consistent correlation of *V. harveyi* inhibition when treated with rearing waters stocked with tilapia or shrimp with tilapia. It is likely that the varying conditions in the sampled ponds at different sites, influenced the results. Therefore, the experiment needs repetition under more controlled culture conditions.

GREEN WATER CULTURE: BACTERIAL, FUNGAL AND ALGAL POPULATION/FLORA

The microbial and phytoplankton flora associated with the green water culture system of *P. monodon* were estimated and isolated. Bacterial counts using the spread plate method were done on nutrient agar (NA) supplemented with NaCl, thiosulfate citrate bile salts (TCBS) agar and Pseudomonas Aeromonas Selective Agar (GSP). Identification was based on Gram staining characteristics and biochemical tests following Baumann *et al.* (1984), Krieg (1984) and Popoff (1984). Fungal enumeration, isolation and identification followed the methods of Barnett and Hunter (1987), Kohlmeyer and Volkman-Kohlmeyer (1991) and Leaño (2002). Phytoplankton estimates were made using standard methods on a Burker Turk hemacytometer and a Sedgwick-Rafter counting chamber with characterization. Identification of the algae followed the key of Yamaji (1991).

The total bacterial counts (TBC) of cultivable bacteria in rearing water from tilapia pond, shrimp with tilapia and seawater sources in Negros Occidental did not vary significantly. The estimated TBCs were 10^2-10^3, 10^2-10^4 and 10^2-10^3 cfu/ml at days of culture (DOC) 15, 30, 45 and 60, respectively. The highest bacterial count of 10^4 cfu/ml was only observed in the shrimp with tilapia culture waters. The hepatopancreas of shrimps cultured with tilapia had TBCs of 10^3 to 10^5 cfu/g. In the tilapia mucus, an increasing trend in TBCs of 10^3, 10^4, 10^4 and 10^5 cfu/5 cm^2 at DOC 15, 30, 45 and 60, respectively, were observed. The tilapia gut, on the otherhand, yielded TBCs of 10^5-10^6 cfu/5 cm.

Likewise, the presumptive *Vibrio* counts (PVC) of water sources and the green water rearing waters did not vary significantly at 10-10^3 cfu/ml. The PVCs of the hepatopancreas of shrimps cultured with tilapia were 10-10^2 cfu/g. The mucus of tilapia contained PVCs of 10^2-10^4 cfu/ 5 cm^2 while the fish gut harbored PVCs of 10^4-10^6 cfu/5 cm.

The luminous bacteria counts (LBC) were detectable at a range of 10-10^2 cfu/ml in the water sources but not in the rearing waters. All sampled hepatopancreas of shrimp cultured with tilapia had no detectable luminous bacteria. There were also no luminous bacteria in the tilapia mucus but the tilapia gut had LBCs of 10-10^5 cfu/5 cm.

By and large, the bacterial flora associated with the "green water culture" rearing waters consisted of non-luminous *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp, *Flavobacteria* spp., members of the Enterobacteriaceae family and some unidentified bacteria at varying percentages (Lio-Po *et al*., 2001).

On the other hand, total fungal flora showed the highest load on the fish gut at 10^2-10^3 cfu/5 cm and mucus samples at 48-10^2 cfu/5 cm^2. Lower fungal load, however, were observed in shrimp hepatopancreas and water samples yielding 2-37 cfu/g and 5-44 cfu/ml, respectively. The yeast population was dominated by *Rhodotorula* sp., *Saccharomyces* sp. and *Candida* sp. Among the genera of filamentous fungi commonly observed were *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Curvularia* sp.

The phytoplankton flora of the green water culture system predominantly consisted of *Chlorella* at 10^5-10^6 cells/ml followed by an alga tentatively identified as *Leptolyngbia* sp. at 10^4-10^5 cells/ml. Other species like, *Thalassiosira* sp., *Skeletonema* sp., *Nitzchia* sp., *Navicula* sp., *Chaetoceros* sp and *Anabaena* sp. were also detected at lesser concentrations.

GREEN WATER CULTURE: ANTI-*VIBRIO HARVEYI* METABOLITES

Bacterial, fungal and algal isolates obtained from the "green water culture" system were screened for the presence of extracellular and intracellular metabolites affecting growth and viability of luminous *Vibrio.* Pure cultures of each isolate were used. *In-vitro* co-cultivation of selected bacterial and algal isolates with *V. harveyi* were conducted and the survival of *V. harveyi* was quantified after 24 and 48 h exposure. Fungal assays for extracellular metabolites were conducted using the modified disc-agar-diffusion method.

Results showed that of the 111 bacterial isolates tested, 31, 18 and 18 isolates exerted marked, moderate and slight inhibition of *V. harveyi*, respectively, after 24 h culture. Among fungal isolates, no zones of inhibition were observed with the extracellular metabolites of 20 yeast isolates. However, the intracellular extracts of four yeast isolates caused growth inhibition of *V. harveyi.* Among the 41 filamentous fungal isolates, the extracellular metabolites of three isolates caused slight inhibition of the test bacterial pathogen while the intracellular extracts of five isolates showed slight inhibition of the *V. harveyi.* With the algae, *Skeletonema* sp. induced a one-log reduction of *V. harveyi* after 48-72 h incubation with its extracellular metabolites.

DISCUSSION

In this study, the total bacterial counts observed in the hepatopancreas of shrimps in the "green water culture" system at DOC 30 was about two logs less than that observed in *P. monodon* with luminous vibriosis in the Philippines in an earlier study and in intensively cultured

shrimps in Thailand (Leaño *et al*., 1998; Ruangpan *et al*., 1994). The bacterial flora associated with the hepatopancreas of shrimps in the "green water culture" system at DOC 15, 30, 45 and 60 was predominated by non-luminous *Vibrio.* In contrast, Ruangpan *et al.* (1994) reported that *Pseudomonas spp.* predominated over *Vibrio* spp. in the hepatopancreas of intensively cultured *P. monodon* in Thailand. It was also reported that the hepatopancreas of pond-reared *P. monodon* juveniles associated with luminous *Vibrio* outbreaks may harbor *V. harveyi* of as much as 10^6 cfu/g (Leaño *et al*., 1998). These findings indicate that the "green water culture" favorably affects the bacterial population of the rearing waters and of the cultured shrimps.

The present study has shown that some bacteria, fungi and phytoplankton associated with the "green water culture" system secrete growth inhibitory metabolites against *V. harveyi.* The undetectable levels of luminous bacteria in the hepatopancreas of shrimps cultured with tilapia and in the mucus of tilapia is a further positive indicator that the "green water culture" system does prevent the multiplication of the luminous *Vibrio.* This may be attributed to the strong antibacterial activity of protein extracts found in the fish mucus (Ebran *et al*., 1999). Moreover, Chong *et al.* (2002) reported that among tilapia, snakehead and carp mucus, tilapia mucus had the highest level of proteins. Earlier, Torrento and Torres (1996) and Ruangpan *et al*., (1998) reported the *in-vitro* inhibition of *V. harveyi* by *Pseudomonas* spp. and *Vibrio alginolyticus*, respectively.

The presence of filamentous fungi and yeasts especially in the gut and mucus of tilapia in the "green water culture" of *P. monodon*, may also contribute to the inhibition of the growth of *V. harveyi* through the production of intra- and extracellular metabolites, as shown in the metabolite assays in this study. Many marine fungi produce novel compounds and enzymes (Biabani and Laatsch, 1998). Furthermore, fungi are good sources of glucans, a proven immunostimulant for crustacean species (Sung *et al.* 1994; Devaraja *et al*., 1998, Chang *et al*., 2000). Thus, their role or importance in many marine habitats, including aquaculture ponds, need further investigation.

Microalgae as absorbers of carbon dioxide and providers of oxygen, improve water quality in the aquatic environment through oxygenation and filtration. These processes help prevent the occurrence of diseases caused by pathogenic bacteria and fungi (Round, 1973). Moreover, phytoplankton are potential sources of anti-microbial compounds. *Chlorella*, for instance, is used in the preparation of the antibiotic, Chlorellin, against gram positive and gram negative bacteria (Sharma, 1986). *Nitzschia perlea* produces a growth inhibiting antibiotic against *Escherichia coli* (Round, 1973). In a mixed culture experiment of 10^2 cfu/ml *V. harveyi* with either 10^5 cells/ml *Skeletonema costatum* or 10^6 cells/ml *Chaetoceros calcitrans*, the population of the bacterial pathogen was reduced by at least one log but not in the corresponding algal-free filtrates (Lavilla-Pitogo *et al*., 1998b). Furthermore, Naviner *et al.* (1999) partly purified the bioactive compound found in *S. costatum.*

In summary, the effectiveness of the green water culture system for the grow-out culture of *P. monodon* in preventing luminous vibriosis maybe attributed to the presence of bacteria, fungi and algae that secrete *V. harveyi* growth inhibitory factors. In addition, the yeasts flora that are significantly abundant in tilapia, is a probable source of beta-glucan that is known to exert an immunostimulating effect on *P. monodon* against *Vibrio* infections. Also, the tilapia mucus that apparently prevents the colonization of *V. harveyi* on the fish skin, may also shed these anti-*V. harveyi* factors into the rearing waters thereby contributing to the biocontrol of this bacterial pathogen. It is likely that the low to undetectable levels of luminous *Vibrio* in the green water

culture system for *P. monodon* is a result of the combined inhibitory effects of factors in the fish mucus and microbial flora of this culture system. Therefore, the microbial flora associated with the "green water culture" system is an excellent source of indigenous microorganisms with probiotic potentials.

ACKNOWLEDGMENTS

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DEVELOPMENT OF A REGIONAL AQUATIC ANIMAL DISEASE CONTROL SYSTEM

Fish Disease Control Project of SEAFDEC Aquaculture Department

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INTRODUCTION

In the past 10 years, aquaculture production in Southeast Asia has grown rapidly and contributed to worldwide food supply. However, a number of infectious diseases have emerged, with the rapid and uncontrolled development of aquaculture, threatening the sustainability of aquaculture in the region. Moreover, the widespread use of chemicals including antibiotics to control these diseases can pose a danger to consumers' health and become an obstacle for trading of the cultured produce. It is, thus, urgent to establish effective control measures against infectious diseases, as well as monitor methods for chemical use.

Realizing these needs, the Aquaculture Department of Southeast Asian Fisheries Development Center (SEAFDEC AQD) with the support of the Japanese Government Trust Fund, initiated the Regional Fish Disease Control Project titled "Development of Fish Disease Inspection Methodologies for Artificially Bred Seeds" from 2000-2003.

The Project consists of the following activities:

1. Research to

   1.1 develop standardized diagnostic methods for the region;
   1.2 develop disease control husbandry techniques; and
   1.3 develop a monitoring method for residual chemicals in aquaculture products.

2 International workshop for the harmonization and standardization of diagnostic method as well as disease control husbandry methods;

3. Hands-on training on diagnostic methods for important diseases in the region; and

4. Development of a disease control network in the region.

RESEARCH AND DEVELOPMENT

At the start of the Project in 2000, research and development activities were conducted solely by SEAFDEC AQD. In order to make the Project more efficient and more relevant to the

region, three agencies of the Department of Fisheries-Thailand were invited to collaborate starting 2001. These are: the Aquatic Animal Health Research Institute (AAHRI), the Marine Shrimp Research and Development Center (MSRDC), and the Samutsakhon Coastal Aquaculture Development Center (SCADC).

The main research activities are as follows:

1. Establishment and standardization of diagnostic methods

Recent pandemics of viral diseases in shrimp as represented by white spot syndrome virus (WSSV) have produced catastrophic economic damage to aquaculture. Similar viral disease impact is also expected for marine fish aquaculture, which has been rapidly growing in the region. The research in this section aims to establish and standardize diagnostic methods for viral diseases, which are practical and applicable in the region. The section also contains some survey on the distribution of important viral diseases in the region.

Shrimp viruses

One study aimed to standardize polymerase chain reaction (PCR) technique as the detection method for WSSV. The study has already refined the extraction methods of DNA and primers to be used. Thus, the technique is ready for the proposed training in the Project. Two studies are tackling the development of new diagnostic methods: one is addressing the production of monoclonal antibody for hepatopancreatic parvovirus (HPV) and monodon baculovirus (MBV); another is establishing a shrimp cell culture system.

A survey is ongoing on the distribution of important viral diseases in wild *Penaeus monodon.* The survey is important in identifying populations of wild shrimp that are free of systemic viruses, since broodstock development of *P. monodon* solely relies on wild stock.

Marine fish viruses

First, we aim to establish virus detection in marine fish at SEAFDEC AQD. Through this Project, the cell lines SBK-2, GF, EPC, FHM, SSN-1, SHS and WSS_2C_1 have been made available. The PCR and immuno-cytochemistry methods for several important viral diseases are also available. These techniques will be utilized for the proposed training under this Project. Using these techniques, viral examination of eggs, larvae and broodstock of various fishes have started. As an output of this activity, we demonstrated by histopathology, PCR and cell culture method using SSN-1 that the mass mortality of grouper larvae observed at SEAFDEC AQD was caused by viral nervous necrosis (VNN). This is the first confirmative identification of VNN in the Philippines. Taking into consideration the impact of VNN, we also began establishing a model system to prevent and control VNN in the hatchery.

In Thailand, a similar survey on viral diseases in grouper is ongoing focusing, on iridovirus and nodavirus, the most devastating viruses in marine fish.

2. Biology and pathogenesis of disease agents

Parasitic infestation has been known to cause mass mortalities in many marine and freshwater fishes. Effective control of parasites necessitates the knowledge of their life cycles, good health management of culture systems of the host fish, and the availability of treatment methods. Thus, a series of studies was conducted in the Philippines and Thailand to screen economically important fishes for the presence of parasites, determine diagnosis and pathology of infections and host-parasite relationships, and establish methods of prevention and control.

Screening for parasites was done in grouper (*Epinephelus coioides*), red snapper (*Lutjanus argentimaculatus*), rabbitfish (*Siganus guttatus*) and catfish (*Clarias macrocephalus*). The most common ectoparasites observed among these fishes were the trichodinids, skin and gill flukes, digeneans, copepods and leech while nematodes were the most common endoparasites. Parallel screenings conducted in 2001 in the Philippines and Thailand on grouper indicated similar parasite fauna.

Healthy grouper fingerlings experimentally exposed to the gill fluke *Pseudorhabdosynochus* sp. resulted in 100% mortality within 72 h of exposure at 5,000 monogeneans/10 fish.

Since heavy gill fluke infestation was found to have caused high level of mortality in grouper, preventive measures were developed. Among the treatments tested, the 1 h bath of 200 ppm H_2O_2 effectively removed the gill parasite.

3. Disease prevention and control

Luminescent vibriosis has long been a major constraint in shrimp culture in the region. Research in this section aims to develop husbandry techniques such as the use of probiotics and the "green water" culture system as alternative strategies for the control and prevention of shrimp vibriosis.

One study assessed the use of live bacteria (probiotics) as a biological control agent against the serious disease luminescent vibriosis and its pathogen, *Vibrio harveyi* in shrimp and crab aquaculture. Tests were conducted to measure growth suppression of the pathogen by competition with antagonistic bacteria in mixed cultures and manipulate gut bacterial flora by introducing benign bacteria through live or inert feeds, or though the water.

Potential probiotic bacteria were isolated from healthy hatchery-reared crustacean larvae and their environments. From the 80 bacterial isolates tested in one-on-one competition experiments, 10 strains were found to suppress the growth of *V. harveyi* within 24 h. One isolate from *Chlorella* sp. culture is considered a promising probiotic for crustacean hatcheries since the bacterium is not pathogenic to crab and shrimp larvae, can associate closely with the larvae and dominate their associated bacterial flora, can readily be incorporated into live *Brachionus plicatilis,* and can easily be cultured in liquid media.

Another study will the evaluate probiotics using a new, experimental method. The method has been refined and ready for evaluation. The candidate probiotics selected in this study as well as commercial probiotics will be evaluated.

The "green water" technique, which transfers rearing water from tilapia culture ponds to shrimp ponds, is also one of the several modifications in shrimp culture to prevent bacterial and viral diseases. Bacterial, fungal and algal isolates associated with the "green water" culture of *P. monodon* were screened for inhibitory metabolites against *V. harveyi.* The results indicated that extracellular and intracellular metabolites of some of the bacteria, fungi and phytoplankton of the "green water" system inhibit the growth of *V. harveyi.*

In a preliminary tank experiment, the existence of tilapia reduced the concentration of *V. harveyi.* This tank experiment is expected to clarify the mechanism of "green water" (or finfish integration) shrimp culture system.

Another study addresses the possible utilization of bacteriophage of *Vibrio* for controlling vibriosis. Samples have been obtained from shrimp hatchery and grow-out farms.

4. Establishment of methods to evaluate residual chemicals in aquaculture products

The presence of chemical residues in aquaculture food products threatens human health. To answer the need for risk-free, nontoxic food, research in this area aims to develop monitoring methods for chemicals in aquaculture products and publication of manuals for the safe and efficient use of chemicals in aquaculture.

Detection of pesticide and antibiotic residues in aquaculture products underwent its first phase of activities. Various extraction techniques for some aquaculture products (shrimps, milkfish, grouper, sea bass, siganid, catfish, tilapia and seaweed) were standardized. The recovery, efficiency, detection limits and reproducibility of methods for 18 pesticides were established.

To collect basic data for guidelines and strategies for antimicrobial usage, a survey on drug resistant-bacteria in shrimp farms in Thailand was conducted. A total of 360 bacterial isolates were collected from 13 places. Among these, 289 were identified as *Vibrio* spp. and 25 as unidentified luminous bacteria. Examination of drug resistance of these bacteria showed that out of 169 isolates, 39 were resistant to oxytetracycline, 6 to oxolinic acid and 2 to chloramphenicol.

COLLABORATION FOR DISEASE CONTROL IN THE REGION

Possible networking schemes for disease control in the region and collaborative schemes for regional diagnosis are shown as Figures 1 and 2. SEAFDEC AQD will collaborate with other international organizations such as OIE, FAO and NACA, focusing on development and dissemination of disease diagnosis and prevention and control methods and on network formation in research activities.

TRAINING

During the *S,minarFWorHshop on Disease Control in Fish and Shrimp Aquaculture in Southeast Asia: Diagnosis and Husbandry Technique*, prioritization of the diseases and diagnostic methods in the region, as well as a training program was discussed. Based on this output, the Project plans to conduct a training course in late 2002 on *Diagnosis of Important Viral Diseases of Shrimp and Marine Fish* in the region.

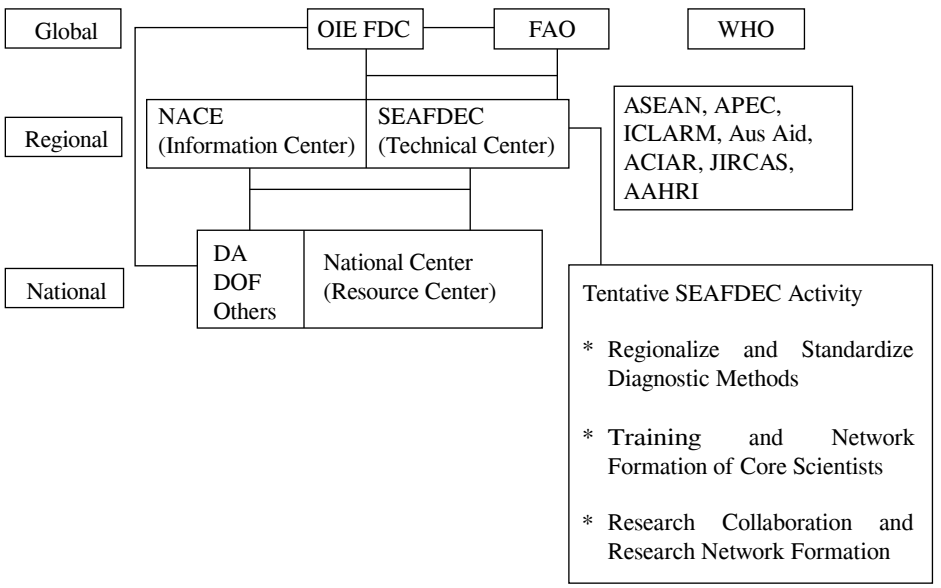

Figure 1. A possible regional network for fish control

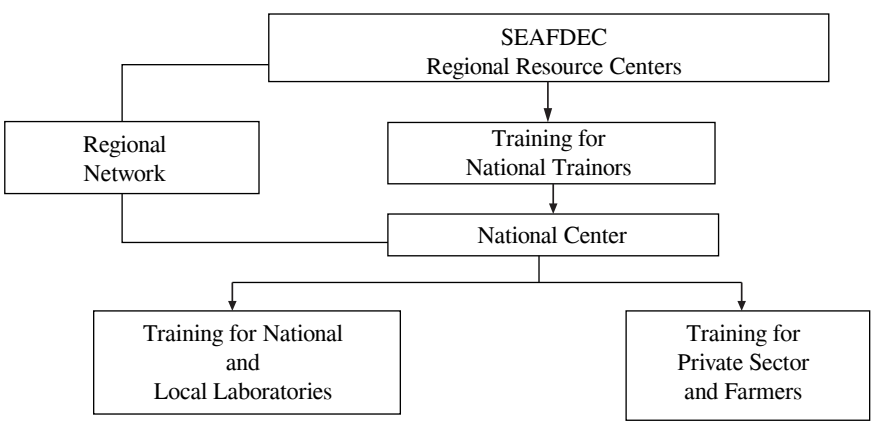

Figure 2. Collaboration scheme for regional diagnosis

Global Aquatic Disease Control Activities of OIE and the Fish Diseases Commission

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The Office International des Epizooties (OIE), also known as the World Organization for Animal Health, is an intergovernmental veterinary organization established in 1924 in order to promote world animal health. Its Central Bureau is based in Paris. One of its main activities is to provide guidelines and standards for health regulations applicable to international trade in live animals and their products. Among the main objectives of the OIE is to increase general awareness of disease problems associated with trade in live animals and animal products, including aquatic animals, and to promote means for diagnosis, control or prevention. These objectives generate an approach based upon the following: coordination of investigations of communicable animal diseases for which international cooperation is essential; collection of information on epizootics and control measures applied by the Member Countries; and an advisory role in preparing international standards or agreements pertaining to animal health. The communication of animal health information to Member Countries occurs through their respective Veterinary/Animal Health Services, although in some Member Countries, another Authority, rather than the National Veterinary Services, is responsible for aquatic animal health. Good communication and cooperation between such different national authorities within a country is important.

The OIE established the Fish Diseases Commission (FDC) in 1960 to deal specifically with the increase of fish diseases as aquaculture expanded worldwide. From 1988, the scope of the FDC was extended to include diseases and pathogens of molluscs and crustaceans. The OIE approach to animal health control in aquaculture involves making recommendations to Member Countries to apply the following measures:

- assessment of the health status of aquatic animals in a production site, based upon inspections and standardized sampling procedures followed by laboratory examinations conducted in accordance with the instructions given in the OIE *Diagnostic Manual for Aquatic Animal Diseases*;
- restocking of open waters and fanning facilities with products of a health status higher than, or equal to, that of the area concerned;
- eradication of diseases of socio-economic importance whenever possible; and
- notification by every Member Country of additional requirements, in addition to those provided by the *Aquatic Code*, for the importation of aquatic animals and aquatic animal products.

If the above procedures are used, it becomes possible to define the health status of aquaculture animals and products for specified pathogens, according to the country, zone or

production site of origin. The health status of the product can thus be warranted by the issue of a health certificate by the appropriate official, stating that the aquaculture products in a defined consignment originate from a country, zone or farm/harvesting site free of the specified pathogens listed in the *Aquatic Code* and possibly of other specified diseases. Aquatic animal diseases included in the OIE system are classified into one of two lists ('notifiable diseases' and 'other significant diseases') on the basis of their socio-economic importance, geographic range and aetiology.

The OIE control policy is thus based on regulations focused on certain diseases, leading to certification of acceptable sources of aquaculture products for national and international trade. The origin is considered as either entire country, zone or protected facility, demonstrated to be officially free of these pathogens, through the implementation of a national health surveillance scheme that employs sampling and laboratory techniques described in the *Manual.*

Both the *International Aquatic Animal Health Code* and *Diagnostic Manual for Aquatic Animal Diseases* are updated annually. Member Countries may propose changes through their Chief Veterinary Officers who communicate directly with the OIE. The proposed changes are examined by the Fish Diseases Commission and draft recommendations are prepared for consideration by Member Countries at the annual General Session held in Paris each year.

New occurrences of diseases in a previously free region must be reported to the OIE in accordance with the reporting requirements of the OIE. The urgency of dispatching information varies according to the nature of the disease. The OIE has devised a warning system whereby Member Countries can take action rapidly should the need arise: countries are required to notify the Central Bureau within 24 hours of the occurrence of an outbreak of a notifiable aquatic animal disease, or any other contagious disease likely to have serious repercussions on public health or the economy of animal production (including aquatic animal production). The OIE immediately dispatches these data by telex, telegram, fax or electronic mail directly to Member Countries at risk, and in weekly announcements (in *Disease Information*) to other countries. In addition to the 'alert' system, information received from Member Countries is distributed on a periodical basis:

- the monthly Bulletin provides data on the course of notifiable diseases month by month. The *Bulletin* also contains sections devoted to the epidemiology and control of the principal contagious diseases and to the activities of the OIE; and
- the annual World Animal Health provides yearly statistics for the OIE notifiable aquatic animal diseases, giving data on the occurrence of diseases in each Member Country, and annual animal health status reports for all Member Countries. These summarize control methods adopted by each country.

It is the task of the FDC to assist Member Countries to overcome limitations related to implementing the above aspects. First, this is done by increasing general awareness of the role and activities of the OIE in the health control of aquatic animals. The OIE has published a brochure describing the aims and objectives of the FDC and there is an FDC web page on the main OIE web site providing information on the work of the FDC as well as making the Aquatic Code and Diagnostic Manual freely available on-line and giving news of any recent important developments in the occurrence of OIE-listed aquatic animal diseases worldwide. There are

useful links to other web sites dealing with aquatic animal health issues, including the International Database on Aquatic Animal Diseases which provides data on the occurrence by country and host of all the OIE-listed diseases of fish and shellfish. There is also active FDC participation in educational programmes to facilitate training of specialists in health problems encountered in aquaculture: members of the Fish Diseases Commission have been involved in several training programmes on aquatic animal diseases in several countries.

Another important aspect is to gather scientific information enabling more acceptable methods of aquatic animal disease therapy, which has been increasingly threatened by restrictive regulations, due not least to media campaigns on residues in fanned aquatic animals. This impact was a major theme at the OIE International Symposium on Chemotherapy in Aquaculture in 1991 and the proceedings were published by OIE in 1992. The problem of disease transfer by international trade in aquatic animals was discussed in depth at the International Conference on Preventing the Spread of Aquatic Animal Diseases through International Trade held in Paris in June 1995; the papers presented and discussions held at this Conference were published in the OIE *Scientific and Technical Review* in June 1996. Since risk analysis has become more and more important in the international trade of live aquatic animals and animal products, the FDC has decided to increase its involvement in this area. The International Conference on Risk Analysis in Aquatic Animal Health held at OIE in February 2000 is one aspect of this endeavour; the proceedings of this conference have also recently been published.

Finally, an effort is being made to further promote cooperation with other international organizations (*e.g.* the European Union [EU], the Food and Agriculture Organization [FAO] of the United Nations and the Network of Aquaculture Centres in Asia and Pacific Region [NACA]) involved in health surveillance and disease control policies in aquaculture so that common agreements are reached, resulting in a merging of the various approaches.

Recent Asian Initiatives Under the NACA Regional Programme on Aquatic Animal Health Management

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ABSTRACT

The activities of NACA in support of improving aquatic animal health management within Asia dates back since 1986 when it was first involved in the UNDP/FAO/ODA (and subsequently DFID) sponsored program on Epizootic Ulcerative Syndrome (EUS). Consequently, in cooperation with relevant governments and institutions, NACA implemented a Regional Research Program on Ulcerative Syndrome in Fish and the Environment, from 1986 to 1989, which produced most of the scientific data on environmental parameters associated with EUS outbreaks in the Asia-Pacific region. Between 1989-1990, NACA and ADB implemented the Regional Study and Workshop on Fish Disease and Fish Health Management which revealed a scenario of environment-linked disease problems, product contamination, and environmental impacts on aquaculture, and for the first time losses suffered by Asian aquaculture from fish diseases were quantified. The study provided the first broad guidelines to regional and national strategies for developing capacities in fish health management. In 1991, OIE Tokyo approached NACA to initiate cooperation with respect to aquatic animal disease reporting which eventually led to an Expert Consultation on Aquatic Animal Disease Reporting in 1996. Between 1992 to 1996, NACA was involved in the following regional activities: (a) collaborating with IDRC and UPM in a Tropical Fish Health Management course, that ran for two intakes of students at UPM; (b) participating in the FAO 1994 Expert Consultation on Health Management held at UPM in Malaysia; and (c) the 1996 Consultation on Quarantine and Health Certification of FAO and AAHRI through the ODA-funded SEAADCP project. Inl998, a joint publication - 'EUS Technical Handbook' with ACIAR, DFID, NSW Fisheries, AAHRI through SEAADCP and NACA - was completed.

The major recommendations of the various regional meetings/consultations became the basis for the development of a strong multi-disciplinary Asia-Pacific regional programme on aquatic animal health management. At the request of Asian governments, NACA and FAO developed a Regional Technical Cooperation Programme on "Assistance for the Responsible Movement of Live Aquatic Animals" (FAO RTCP/RAS 6714 and 9605). The project was implemented from 1998 to 2001 in cooperation with 21 governments/territories in Asia-Pacific region, OIE FDC, OIE Tokyo, AFFA, AusAID/APEC and AAHRI.

The programme and its outputs were developed through three years (1998 to 2001) of awareness raising and consensus building through various national and regional level activities (*e.g.* workshops, training courses, expert consultation, health assessments, etc.). This multi-disciplinary Regional Aquatic Animal Health Management Programme has now been adopted by

Asian governments (including NACA members and participating governments within ASEAN) as an important element of NACA's Third Five Year Work Programme (2001-2005). The current thrust of the programme is to assist countries in implementing the 'Technical Guidelines', giving special emphasis to the concept of "*phased implementation based on national needs*", including monitoring and evaluation of its implementation. One of the mechanisms to support Asian governments in the implementation of the 'Technical Guidelines' is through regional cooperation where effective partnership with relevant organizations will be continuously established and strengthened. Designated National Coordinators will continue to be the focal points for its implementation.

A Regional Advisory Group on Aquatic Animal Health has been established which will function as an official regional expert group that will ensure the provision of expert advice to Asian governments in the implementation of the 'Technical Guidelines', with NACA providing institutional support and FAO and OIE providing technical guidance. The main elements for regional cooperation include: (a) Promoting effective cooperation through regional resource centers on aquatic animal health; (b) Harmonization of procedures for health certification, quarantine and diagnostics; (c) Support to capacity building; (d) Awareness raising, communication and information exchange on aquatic animal health; (e) Regional disease reporting; (f) Emergency response; and (g) Joint activities for risk reduction in shared watersheds.

The paper also briefly include other health related projects jointly being developed and/ or currently carried out by NACA with other organizations (*e.g.* ACIAR, APEC, ASEAN, CSIRO, DANIDA, IDRC, MPEDA, MRC and SEAFDEC-AQD).

INTRODUCTION

The Network of Aquaculture Centres in Asia-Pacific (NACA) is an inter-governmental organization of 15 member governments (*i.e.*, Australia, Bangladesh, Cambodia, China PR, Hong Kong China, India, Korea DPR, Malaysia, Myanmar, Nepal, Pakistan, the Philippines, Sri Lanka, Thailand and Vietnam), including a number of actively participating countries such as Indonesia, Iran, Korea RO, Lao PDR and Singapore. NACA's vision is to assist member and participating governments to improve opportunities for sustainable aquaculture development and aquatic resources management in order to contribute to social and economic development in the Asia-Pacific region. Aquatic animal health management and disease control is a priority element of NACA's Third Five Year Work Programme (2001-2005). NACA's activities on aquatic animal health, however, dates back as far back as two decades ago.

This paper provides chronological information about NACA's aquatic animal health management program - a brief history, recently concluded projects particularly the FAO TCP/RAS 6714 and 9605 "Assistance for the Responsible Movement of Live Aquatic Animals" and current and forthcoming projects.

BRIEF HISTORY OF NACA BEGINNINGS ON AQUATIC ANIMAL HEALTH MANAGEMENT

NACA implemented a Regional Research Program on Ulcerative Syndrome in Fish and the Environment, from 1986 to 1989, under the cooperative framework of UNDP/FAO/ODA. The program produced most of the scientific data on environmental parameters associated with the outbreak of Epizootic Ulcerative Syndrome (EUS) in the Asia-Pacific region (Roberts *et al*., 1986; Phillips, 1989; Phillips and Keddie, 1990). In 1992, the Aquatic Animal Health Research Institute (AAHRI) of Thailand's Department of Fisheries and NACA jointly published a review of the EUS which contained much of the literature on the subject from both national and international articles, reports and conference proceedings (Lilley *et al*., 1992). Subsequently, a joint publication - 'EUS Technical Handbook' with the Australian Centre for International Agricultural Research (ACIAR), the Department of International Development (DFID) of the United Kingdom, the New South Wales Fisheries (NSW Fisheries) of Australia, AAHRI through the Southeast Asia Aquatic Animal Disease Control Project (SEAADCP) and NACA - was completed. This handbook contains additional practical applications to assist in the diagnosis and control of EUS (Lilley *et al.* 1998). NACA was also involved in a study visit to investigate the occurrence of EUS in Pakistan. This was undertaken with AAHRI, ACIAR and Institute of Aquaculture of Stirling University.

Between 1989-1990, NACA implemented an ADB-funded Regional Study and Workshop on Fish Disease and Fish Health Management which revealed a scenario of environment-linked disease problems, product contamination, and environmental impacts on aquaculture, and for the first time losses suffered by Asian aquaculture from fish diseases were quantified. The study provided the first broad guidelines to regional and national strategies for developing capacities in fish health management (ADB/NACA, 1991).

In 1991, the Office International des Epizooties (OIE) Regional Representation for the Asia-Pacific based in Tokyo, Japan (OIE Tokyo) approached NACA to initiate cooperation on aquatic animal disease reporting. The same year a representative of OIE Tokyo participated at the NACA's Governing Council Meeting (GCM). This eventually led to an Expert Consultation on Aquatic Animal Disease Reporting in 1996 with representatives from OIE Tokyo, OIE Fish Disease Commission (OIE FDC) and a number of selected aquatic animal health experts in the region.

The period between 1992-1996 saw NACA involved in more regional activities. These were (a) collaboration with the International Development Research Centre (IDRC) of Canada and the Universiti Pertanian Malaysia (UPM) in a Tropical Fish Health Management course, and supported two batches of Asian students; (b) participation in the FAO 1994 Expert Consultation on Health Management held at UPM; and (c) co-organization of the 1996 Consultation on Quarantine and Health Certification of FAO and AAHRI through the ODA-funded SEAADCP project (Humphrey *et al.* 1997).

The major recommendations of the various regional meetings/consultations and activities mentioned above became the basis for the development of a strong multi-disciplinary Asia-Pacific regional programme on aquatic animal health management. The 1996 FAO Consultation on Quarantine and Health Certification, particularly catalyzed the development of an FAO Regional Technical Cooperation Programme described in the following section.

FAO REGIONAL TECHNICAL COOPERATION PROGRAM (TCP/RAS 6714 AND 9605) PROJECT "ASSISTANCE FOR THE RESPONSIBLE MOVEMENT OF LIVE AQUATIC ANIMALS"

At the request of 15 Asian governments, NACA and FAO developed a Regional Technical Cooperation Programme on "Assistance for the Responsible Movement of Live Aquatic Animals" (FAO RTCP/RAS 6714 and 9605). The project was participated by 21 governments/territories in Asia-Pacific region (*i.e.*, Australia, Bangladesh, Cambodia, China PR, Hong Kong China, India, Indonesia, Iran, Japan, Korea DPR, Korea RO, Lao PDR, Malaysia, Myanmar, Nepal, Pakistan, Philippines, Singapore, Sri Lanka, Thailand and Vietnam), and supported by a number of regional and international organizations and agencies such as OIE Tokyo, OIE FDC, Australia's Agriculture, Fisheries and Forestry (AFFA), the Australian Agency for International Development - Asia Pacific Economic Cooperation (AusAID/APEC) and AAHRI.

The objective of the program was to develop technical guidelines on health management and responsible movement (introductions and transfers) of live aquatic animals, through appropriate strategies that minimize potential health risks associated with live aquatic animal movements; and that which are in concordance with other international agreements and treaties (WTO's Sanitary and Phytosanitary Agreement or SPS Agreements and OIE's health standards - the Code and the Diagnostic Manual), in support of FAO's Code of Conduct for Responsible Fisheries (CCRF), and that which is practically applicable to the Asian region.

Program implementation and further development took three years (1998 to 2001) of awareness raising and consensus building through various national and regional level activities *(e.g.* workshops, training courses, expert consultation, health assessments, etc.). National Coordinators of each of the 21 participating governments were designated and they became the focal persons of the participating countries on aquatic animal health. A Regional Working Group (RWG) and Technical Support Services (TSS) composed of aquatic animal health experts, within the region and outside were formed to assist in the over-all implementation of the program (Anonymous, 1998).

The major outputs of the program are:

(a) Regional Strategy on Responsible Movement of Live Aquatic Animals

The Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals and the Beijing Consensus and Implementation Strategy" or 'Technical Guidelines' (FAO/NACA, 2000) contains the guiding principles that will assist countries in undertaking responsible movement of live aquatic animals in a way that minimizes the risks of trans-boundary pathogens/diseases being introduced and spread with the movement (introduction and transfers) of their hosts. The 'Technical Guidelines' contains the following elements: guiding principles, pathogens, disease diagnosis, health certification and quarantine measures, disease zoning, disease surveillance and reporting, contingency planning, import risk analysis, national strategies and regulatory frameworks, capacity building and an implementation strategy.

The 'Technical Guidelines' is accompanied by an Implementation Strategy - the Beijing Consensus and Implementation Strategy - which gives special emphasis to the concept of "*phased implementation based on national needs*" and two other supporting technical documents: (i) the Manual of Procedures for the Implementation of the Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals (FAO/NACA, 2001) and (ii) the Asia Diagnostic Guide to Aquatic Animal Diseases (Bondad-Reantaso *et al*., 2001). These key publications which contain the regional strategy are in accordance with the WTO's SPS Agreement and the OIE aquatic animal health standards and the first of a regional technical guidelines supporting the implementation of FAO's CCRF.

Adopted in principle by the 21 participating governments/territories during a final workshop in Beijing, China PR in June 2000, the 'Technical Guidelines', also received strong support from the Association of South East Asian Nations (ASEAN) which endorsed the 'Technical Guidelines" as an ASEAN policy document during the 9th Meeting of the ASEAN Working Group on Fisheries held in September 2001 in Bali, Indonesia. Consequently, the 'Technical Guidelines' will be discussed at the next Senior Officials Meeting of ASEAN and subsequently in the Ministerial Meeting of ASEAN in 2002.

The ASEAN-SEAFDEC Fisheries Consultative Group (FCG) during the development of the 'Regional Guidelines for Responsible Fisheries in Southeast Asia - Responsible Aquaculture' held in Iloilo, Philippines in July 2001 endorsed the provision of support for the implementation of the 'Technical Guidelines.' The major references are:

(a) Article 9.3.2 "States should cooperate in the elaboration, adoption, and implementation of international codes of practice and procedures for introductions and transfers of aquatic organisms." The two relevant statements are:

4) *"States should support the implementation of the 'Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals and the Beijing Consensus and Implementation Strategy' with emphasis on phased implementation based on national needs"*; and

5) *"The National Strategies on Aquatic Animal Health Management in the 'Technical Guidelines' should be integrated into the national aquaculture development plans of States in the region. States should provide funds for its implementation."*

Support to the implementation of the 'Technical Guidelines' was again re-emphasized during the ASEAN-SEAFDEC Millenium Conference "Fish for the People" held in Bangkok, Thailand on 19-24 November 2001, and was included as one of the major recommendations and action plans under Session 3.4 - Healthy and Wholesome Aquaculture (Anonymous, 2002).

The Asia Pacific Economic Cooperation (APEC) Marine Resources Conservation Working Group (APEC MRCWG) during a meeting held in Hobart, Australia, in November 2001, recommended closer consideration of instruments, guidelines and processes (including the 'Technical Guidelines') relevant to introduction of marine pests.

The 'Technical Guidelines' also provided the technical basis for the approval and/or development of a number of other regional projects on aquatic animal health. Examples include the following: (a) APEC Fisheries Working Group (APEC FWG) 02/2000 "Development of a Regional Research Programme on Grouper Virus Transmission and Vaccine Development" (APEC/AAHRI/FHS-AFS/NACA, 2001); (b) APEC FWG 03/2000 "Joint APEC/FAO/NACA/SEMARNAP Workshop on "Trans-boundary Aquatic Animal Pathogen Transfer and the Development of Harmonized Standards on Aquaculture Health Management" (APEC/FAO/NACA/SEMARNAP, 2001); (c) APEC FWG 2002 "Capacity and Awareness Building on Import Risk Analysis (IRA) for Aquatic Animals" - for implementation in 2002; and (d) Mekong River Commission's (MRC) Mekong Basin Aquatic Animal Health Management Programme which is still under development phase.

(b) Asia-Pacific Quarterly Aquatic Animal Disease (QAAD) Reporting System

The reporting system for aquatic animal diseases was developed following the recommendations of the NACA/OIE Expert Consultation in 1996 and was eventually integrated into the Regional Programme. It commenced in 1998, jointly implemented with OIE Tokyo and OIE FDC, and involves a list of diseases of finfish, molluscs and crustaceans identified to be important to the region together with the OIE list of notifiable and other significant diseases. To-date, 13 quarterly issues have been published and the reporting system is continuing with long-term financial assistance granted by the NACA Member Governments and technical assistance by NACA staff (NACA/FAO, 1999).

Through this quarterly reporting system, there is now emerging a clearer, up-to-date health profile for these diseases within the Asian region which can be used for initiating control and eradication as well as early warning, contingency plans and emergency-preparedness programs. Although still at its very early stages of development, surveillance and reporting systems will in the long run become a 'value added' label to aquaculture and fisheries products because they reflect a country's commitment and ability to collect and provide documented information on the health, origin and quality of each commodity. Countries with a sound aquatic animal health infrastructure and a demonstrated record of surveillance, containment and disease control programs provide them a significant trade advantage.

The QAAD was revised and updated in November 2001 and beginning the first quarter reporting period for 2002, an updated list of diseases based on the 4th edition of OIE International Code for Aquatic Animals (see **http://www.oie.int**) will be reported, with an additional column to reflect the levels of diagnosis (*i.e.*, Level I, II or III - see Table 1 for explanatory notes) for the disease reports as agreed upon during the Provisional Meeting of the Regional Advisory Group on Aquatic Animal Health. The deadline for submission of reports has also been extended (2.5 months from end of each reporting period).

Table 1. Diagnostic levels, associated requirements and responsibilities

Level-Activities	Work Requirements	Responsibility	Technical requirements to support activities
Level I Activities Observation of animal and the environment Clinical examination	Knowledge of normal (feeding, behaviour, growth of stock, etc.); Frequent/regular observation of stock Regular, consistent record-keeping and maintenance of records - including fundamental environmental information Knowledge contacts for health diagnostic assistance Ability to submit and/or preserve representative specimens for optimal diagnosis (Levels II, III)	Farm worker/manager Fishery extension officers On-site veterinary support Local fishery biologists	Field keys Farm record keeping formats Equipment list Model clinical observation sheets Pond-side check list Preservation/transportation guidelines for Levels II/III diagnoses Model job descriptions/ skill requirements Asia Diagnostic Guide to Aquatic Animal Diseases
Level II Activities Parasitology Bacteriology Mycology Histopathology	Laboratories with basic equipment and personnel trained/experienced in aquatic animal pathology Keep and maintain accurate diagnostic records Preserve and store specimens Knowledge of/contact with different areas of specialization within Level II; Knowledge of who to contact for Level III diagnostic assistance	Fish biologists/Technicians Aquatic veterinarians Parasitologists/Technicians Bacteriologists/Technicians Mycologists/Technicians Histopathologists/Technicians	Model laboratory record-keeping system Protocols for preservation/ transport of samples to Level III Model laboratory requirements/equipment/ consumables lists Model job descriptions/ skills list Contact information for accessing Level II and Level III specialist expertise Asia Diagnostic Guide to Aquatic Animal Diseases OIE Diagnostic Manual for Aquatic Animal Diseases Regional General Diagnostic Manuals
Level III Activities Virology Electron microscopy Molecular biology Immunology	Highly equipped laboratory with highly specialized and trained personnel Keep and maintain accurate diagnostic records Preserve and store specimens Maintenance of contact with people responsible for sample submission	Virologist/Technician Ultrastructural histopatho-logist/Technicians Molecular biology scientists/ technicians	Model laboratory requirements, equipment, consumables lists Model job descriptions/ skills requirements Contact information for reference laboratories Protocols for preservation of samples for consultation/ validation ODE Diagnostic Manual for Aquatic Animal Diseases General molecular and microbiology diagnostic references Asia Diagnostic Guide to Aquatic Animal Diseases

The reporting system paved the way for a more strengthened co-operation and collaboration among FAO, NACA and OIE whereby functional linkages and co-operation between fisheries and veterinary authorities of participating countries are now being established. An '*In-session Paper*' was presented during the meeting of the OIE Regional Commission for Asia, Far East and Oceania, held in Kathmandu, Nepal in November 2001. The paper raised important issues regarding QAAD. As a result, the Meeting made the following recommendations: (a) quarterly reports submitted to the OIE Regional Office in Tokyo should be consistent with annual submissions to OIE Central Bureau; (b) OIE reporting should be done accurately; (c) and that OIE National delegates should cooperate and consult more closely with their national fisheries authorities especially in those countries where jurisdictional responsibility for aquatic animals does not, or not exclusively, lie with veterinary authorities.

Current efforts are now geared towards improving the quality of the reports, and establishing surveillance and monitoring programs for the list of diseases being reported.

(c) Aquatic Animal Pathogen and Quarantine Information System (AAPQIS-Asia)

AAPQIS, an internet-based information systems which enables comprehensive tracking and reporting of parasites and diseases on a regional basis, can serve as an important science-based instrument in making risk assessments for the movement of live aquatic animals. AAPQIS-Asia is based at NACA and is accessible at http://www.enaca.org. AAPQIS-Asia currently contains about 2000 bibliographic data and information on about 50 pathogens, mainly of trans-boundary importance, pathogen records for 22 countries, 100 hosts and 50 color images. More information, pathogens, records, images and drawings will be added, as they become available and shall be updated on a regular basis.

FAO is continuing the further development of AAPQIS to extend to other aquaculture regions of the world, with the development of AAPQIS-Africa, AAPQIS-Latin America and AAPQIS-Mediterranean. In addition, the further development of AAPQIS is being considered under a proposal being developed by the World Fish Center (ICLARM - International Center for Living Aquatic Resources Management) on Aquatic Animal Diseases, Food Safety and Trade for inclusion in the CGIAR Challenge Program on Animal Diseases, Food Safety and Trade.

(d) National Strategy on Aquatic Animal Health Management

Countries who have participated in the regional program are at different stages of development of the 'National Strategy' that contains the action plans of governments and which form the basis for the national level implementation of the 'Technical Guidelines'. The 'National Strategy' has the following components: legislation/policy, national coordination, list of pathogens, institutional resources, diagnostics, disease zoning, surveillance/reporting, contingency planning, import risk analysis, capacity building, and private sector consultation. These strategies are expected to be incorporated into the aquaculture development programs of participating countries. Box 1 shows some brief highlights with respect to 'National Strategy' development of some of the participating governments.

Box 1: Brief highlights of national strategies on aquatic animal health

Australia: Australia has a National Strategic Plan for Aquatic Animal Health 1998-2003 - AQUAPLAN - in place since April 1999. The Fish Health Management Committee (FHMC) ministerially appointed, is the body which oversees the development and implementation of AQUAPLAN. AQUAPLAN, which served as a model for other participating countries, is a broad, comprehensive strategy that outlines objectives and projects to develop a national approach to emergency preparedness and response and to the overall management of aquatic animal health in Australia. It comprises eight key programs under which Australia's government and private sectors have identified priority projects to achieve the program objectives. These are: (a) international linkages, (b) quarantine, (c) surveillance, monitoring and reporting, (d) preparedness and response, (e) awareness, (f) research and development, (g) legislation, policies and jurisdiction, and (h) resources and funding. Under the program, the following documents have been released: (a) Australian Aquatic Animal Disease Identification Field Guide (March 2000); (b) AQUAPLAN Zoning Policy Guidelines (August 2000, January 2001)); (c) AQUAVETPLAN Enterprise Manual (December 2000); and (d) AQUAVETPLAN Furunculosis Disease Strategy Manual (June 2001).

India: India completed two consultative meetings (May and November 2001) to develop India's National Strategic Plan for Aquatic Exotics and Quarantine. The consultation was coordinated by the National Bureau of Fish Genetics and Resources and attended by all national ICAR Directors, fish health experts, policy makers, and other relevant organizations and institutes. The strategic plan comprise of 3 documents: (a) a strategic plan; (b) quarantine guidelines, and (c) handbook on exotics and quarantine. The strategic plan is ready for submission to the Ministry of Agriculture for implementation at the national level.

Indonesia: With the ministry level reorganization completed and a Directorate on Fish Health Management and Environment created, a national workshop will be convened in year 2002 to update the National Strategy that was developed in April 2000 with a new set of organizational responsibilities.

Myanmar: A national workshop will be convened in April 2002 to further develop the National Strategy and develop a proposal to source out funding for its implementation.

Nepal: A national workshop was convened from December 12-14, 2001 in Kathmandu, Nepal, to update the National Strategy and develop a proposal to source out funding for its implementation.

Philippines: A national workshop was convened in January 2002 involving key personnel of the Philippine Bureau of Fisheries and Aquatic Resources to further develop the 'National Strategy.' In this workshop, the different elements were prioritized and follow-up action plans with clear time-frame were identified and will be subject to further consultation with all relevant stakeholders before approval by the highest authority.

Singapore: Singapore's National Strategy is currently looking at import risk assessment and plans to monitor disease occurrence in newly introduced species for a period of three months is being considered. The Agri-food and Veterinary Authority of Singapore (AVA) continues to review and update the national list of diseases in order to prioritize health management actions for important marine foodfish pathogens identified. The AVA continues to hold dialogues with stakeholders and to bring awareness of the 'Technical Guidelines' and other health related issues.

Thailand: Thailand's National Strategies for Fish Disease Research and Development for 2001-2005 is in the process of being finalized. It's mission is to develop roles in controlling disease epizootics, technology for disease prevention and control and information exchange. The plan has 7 elements: (a) law and regulation; (b) organization; (c) cooperation; (d) research development; (e) academic development; (f) scientific services development; and (g) information technology.

Vietnam: A National Workshop on the Development of Vietnam's National Strategy for Aquatic Animal Quarantine was held in Hanoi, Vietnam on October 10-11, 2000. The workshop focused on 5 major areas: (a) quarantine and health certification, (b) diagnostics and health management, (c) aquatic animal health information systems, (d) policy and legislation, and (e) proposal for the implementation of the National Strategy. The National Strategy will be finalized in January 2002 for submission to the Ministry of Fisheries.

(e) National and Regional Capacity Building on Aquatic Animal Health

National capacity building activities through in-country training/workshops on various aspects of aquatic animal health management were undertaken during the three-year project implementation period. More details of national and regional level capacity building exercises are described in Box 2.

Briefly, national workshops concerning 'National Strategy' development including national disease reporting and information systems were held in Bangladesh, China PR, India, Indonesia, Philippines, Thailand and Vietnam. Training on Import Risk Analysis (IRA) was also undertaken in the Philippines and participated by officers from Hong Kong China, Malaysia and Thailand. Health assessment was also implemented in Lao PDR and Vietnam. A Basic Training Course on Health Management was held in Vietnam and attended also by officers from Cambodia and Lao PDR.

At the regional level, the three major workshops were conducted to build consensus and awareness to participating governments. A molluscan health management course, the first of its kind in the region, was initiated and participated by aquatic animal health specialists from Indonesia, Japan, Korea RO, Malaysia, Philippines, Thailand and Vietnam. Two expert technical consultations were held, one looking at the issues relating to research needs for standardization and validation of DNA-based molecular diagnostic techniques for the detection of aquatic animal pathogens and diseases (Walker and Subasinghe, 2000); and another on primary aquatic animal health care in small-scale rural aquaculture development (FAO/NACA/DFID/GOB, 2000).

Box 2. National and regional capacity building activities (1998-2001)

1. In-Country Training/Workshops:

Bangladesh
* National Workshop on Disease Reporting and Development of National Disease Reporting System, November 24-28, 1998, Dhaka, Bangladesh
* Workshop on the Development of National Strategy Framework for Quarantine and Health Certification, April 13-15, 1999, Dhaka, Bangladesh

Cambodia
* Participation of two Cambodians to the Training Course on Disease Diagnosis and Surveillance in Cultured Aquatic Animals, June 7-11, 1999, RIA-1, Hanoi, Vietnam

China PR
* National Workshop on Quarantine, Health Certification, Information and Disease Reporting Systems, July 26-30, 1999, Beijing, China PR

Hong Kong SAR China
* Two health officers attended the Philippine National Training Workshop on Import Risk Analysis (IRA) and Aquatic Animal Disease Surveillance, Reporting and Contingency Planning, November 15-20, 1999, Cebu City, Philippines

India
* Development of Practical Guidelines and Strategy for Aquatic Animal Health Certification and Quarantine for India, May 28-29, 1998, Bhubaneswar, India
* Workshop on the Development of a Reporting System for Aquatic animal Diseases, May 30-31, 1998, Bhubaneswar

Indonesia
* National Workshop on the Development of Indonesia's National Strategy on Aquatic Animal Health, May 22-25, 2000, Jakarta

Lao PDR
* Three participants from Lao PDR participated in the Training Course on Disease Diagnosis and Surveillance in Cultured Aquatic Animals, June 7-11, 1999, RIA-1, Hanoi, Vietnam
* Aquatic Animal Health Assessment Survey in Southern Lao PDR, August 2-13, 1999, Savannakhet, Lao PDR

Malaysia
* Two health officers attended the Philippine National Training Workshop on Import Risk Analysis (IRA) and Aquatic Animal Disease Surveillance, Reporting and Contingency Planning, November 15-20, 1999, Cebu City, Philippines

Philippines
* *National* Training Workshop on Import Risk Analysis. November 15-17, 1999, Cebu City, Philippines
* National Training Workshop on Aquatic Animal Disease Surveillance, Reporting and Contingency Planning, November 18-20, 1999, Cebu City, Philippines

Thailand
* National Training Workshop on Aquatic Animal Disease Surveillance and Reporting, September 2-4, 1999, Bangkok
* One fish health officer attended the Philippine National Training Workshop on Import Risk Analysis (IRA) and Aquatic Animal Disease Surveillance, Reporting and *Contingency* Planning, November 15-20, 1999, Cebu City, Philippines

Vietnam
* Training Course on Disease Diagnosis and Surveillance in Cultured Aquatic Animals, June 7-11, 1999, RIA 1, Hanoi
* Inter-Institutional Meeting on Quarantine, Health Certification and Information Systems for the Responsible Movement of Live Aquatic Animals, June 14-16, 1999, RIA 1, Hanoi, Vietnam
* Training/workshop on aquatic animal health assessment and management of Tam Giang Lagoon. Hue. Vietnam, 19-23 August 1999 and 15-22 April 2000
* Vietnam National Strategy Development Workshop, October 2000, RIA 1, Hanoi

2. Regional Workshops
* First Training Workshop of the Regional TCP, January 16-20, 1998, Bangkok, Thailand
* Second Training Workshop of the Regional TCP, February 1-5, 1999, Bangkok, Thailand
* Final Workshop of the Regional TCP, 26-30 June, 2000, Beijing

3. Expert Consultations/Regional Workshops/Training Courses
* Expert Consultation on the Research Needs for the Standardization and Validation of DNA-based Molecular Diagnostic Techniques for the Detection of Aquatic Animal Pathogens and Diseases, February 7-9, 1999, Bangkok (jointly organized with ACIAR, CSIRO and DFID)
* Scoping Workshop on Primary Aquatic Animal Health Care in Small-Scale Rural Aquaculture Development, September 27-30, 1999, Dhaka (jointly organized with DFID)
* Asia-Pacific Regional Programme on Molluscan Health Management, Phase I: Training Course on Basic Molluscan Health Management, 29 November to 3 December 1999, Tigbauan, Iloilo (jointly organized with SEAFDEC-AQD, OIE, IFREMER, DFO-Canada and NIWA-New Zealand)
* Joint APEC/FAO/NACA/SEMARNAP Workshop on Trans-boundary Aquatic Animal Pathogen Transfer and the Development of Harmonized Standards on Aquaculture Health Management, Puerto Vallarta, Mexico, 24-28 July 2000
* Workshop of APEC FWG 02/2000 "Development of a Regional Research Framework on Grouper Virus Transmission and Vaccine Development", 18-20 October 2000, Bangkok, Thailand (jointly organized with APEC, FHS/AFS and AAHRI)
* Provisional Meeting of the Asia Regional Advisory Group on Aquatic Animal Health (AG), 7-9 November 2001, Bangkok, Thailand

NACA'S THIRD YEAR WORK PROGRAMME (2001-2005) - COMPONENT ON AQUATIC ANIMAL HEALTH MANAGEMENT AND DISEASE CONTROL

The various components identified in the 'Technical Guidelines' are now the main elements of the Aquatic Animal Health Management and Disease Control Programme under NACA's Third Five Year Work Programme (2001-2005), approved during NACA GCM-12 held in Brisbane, Australia in December 2000 and the 2.5 year work activities recently planned and endorsed by the Sixth Meeting of the Technical Advisory Committee of NACA (TAC-6) held in Siem Reap in May 2001. As indicated elsewhere in this paper, support for the implementation of the 'Technical Guidelines' was expressed by APEC, ASEAN, MRC and SEAFDEC.

The main elements of the component on Aquatic Animal Health Management and Disease Control include: (a) promoting effective cooperation through regional resource centers on aquatic animal health; (b) harmonization of procedures for health certification, quarantine and diagnostics; (c) support to capacity building; (d) awareness raising, communication and information exchange on aquatic animal health; (e) regional disease reporting; (f) emergency response; and (g) joint activities for risk reduction in shared watersheds.

Assistance to countries in the implementation of the 'Technical Guidelines,' with special emphasis to the concept of "*phased implementation based on national needs*," including monitoring and evaluation of its implementation, is the current thrust of the health program. Regional cooperation is a key strategy where effective partnerships with relevant organizations will continuously be established and strengthened. The National Coordinators will continue to be the focal points for implementation at the national level.

The first major activity undertaken to move forward the implementation of the 'Technical Guidelines' was the establishment of the Regional Advisory Group on Aquatic Animal Health (AG) during a meeting held at the NACA Headquarters in Bangkok from November 7-9, 2001. The Terms of Reference (TOR) of the AG, composition and follow-up activities for the coming year were finalized. The AG represents an official expert group on aquatic animal health, institutionalized under the NACA inter-governmental organization, with technical assistance from FAO and OIE, who will provide regular expert advice to Asian governments in the implementation of the 'Technical Guidelines.'

CURRENT AND FORTHCOMING PROJECTS

A number of current projects being implemented (and/or under development) by NACA in cooperation with NACA's partners in development are described below:

Shrimp Health Management Training Workshop

This regular training workshop on shrimp health management carried out with AAHRI, lasts for 6 days and includes lectures, practical case studies, and farm visits. The emphasis is on maintaining healthy stock and preventing disease through management. The course is attended primarily by participants from the private sector.

APEC FWG 01/2002 "Capacity and Awareness Building on Import Risk Analysis for Aquatic Animals"

With NACA, as Project Implementor and Thailand's Department of Fisheries as Project Overseer, with Australia, Hong Kong China, Mexico, Philippines and the United States as cooperating economies, this Project consists of two training/workshops (Bangkok, Thailand on 1-6 April 2002; 12-17 August 2002 in Mazatlan, Mexico) whose objectives are to raise capacity among regulatory officers and aquatic animal health scientists, build consensus in conducting import risk analysis for aquatic animal importation, and establish networking. An IRA Manual for Aquatic Animals will also be developed as an outcome of the project. This project is a follow-up of one of the major recommendations from two APEC FWG funded projects where NACA was a collaborator, namely: (a) APEC FWG 03/2000 "Transboundary Aquatic Animal Pathogen Transfer and the Development of Harmonized Standards on Aquaculture Health Management (see APEC/ FAO/NACA/SEMARNAP, 2001); and (b) APEC FWG 02/2000 "Development of a Regional Research Programme on Grouper Virus Transmission and Vaccine Development" (APEC/AAHRI/ FHS-AFS/NACA, 2001).

FAO/NACA Molluscan Health Management Programme

FAO and NACA initiated a programme on molluscan health management in response to the recommendations arising from the Second Workshop of the Regional Programme on Aquatic Animal Health Management regarding the shortage of information and knowledge about molluscan diseases in the region. The workshop considered and recognized the need to establish baseline expertise that will provide the foundation for countries to develop their own national programmes for molluscan health monitoring, disease risk analyses and control of epizootics.

The over-all objectives of the Molluscan Health Management Programme are: (a) train national staff on techniques used in molluscan disease investigation, diagnosis and treatment; (b) build expertise in molluscan disease diagnosis and research in the Asian region; (c) through country-specific mollusc health survey/assessment - identifying molluscan diseases existing in, and of concern, to the Asian region; (d) prepare a Manual on Molluscan Health Management for Asia-Pacific; and (d) establish a network of people with expertise in molluscan diseases.

The programme which is of three phases is expected to: (a) develop baseline data on the health profile of economically significant molluscan species in the region; (b) identify molluscan diseases of concern to the Asian region and assist in updating the list of molluscan diseases included in the Asia-Pacific Quarterly Aquatic Animal Disease Report; (c) develop uniform standards of molluscan disease diagnosis, pathogen detection and pathogen identification comparable to those used by countries with mollusc aquaculture and fisheries; (d) enhance national and regional capability in molluscan health management and good husbandry practices; (e) establish an Asia-Pacific network of people with expertise in molluscan health; (f) prepare a reference Manual on Molluscan Health based on Asian material; (g) establish a reference collection of important molluscan diseases in the Asian region; and (h) identify a regional center for molluscan health.

Phase I - Basic Molluscan Health Management Training Course was implemented in November 1999 in Tigbauan, Iloilo, Philippines, hosted by SEAFDEC AQD in cooperation with OIE and IFREMER (France), NIWA (New Zealand) and DFO of Canada. Upon completion of the training course, the participants conducted a histological survey of diseases of important molluscan species as agreed during Phase I. Phase II - Training Workshop: Evaluation of Country Specific Survey, Manual Preparation and Follow-Up Training on Levels II/III will be held immediately following the Fish Health Section's (FHS) Fifth Symposium on Diseases in Asian Aquaculture (DAA5) in November 2002 to be hosted by the University of Queensland involving the same participants for an extensive histopathological examination of survey materials collected by participants, drafting an outline of the Molluscan Health Manual and follow-up training on Levels II/III.

Under this program, the preparation of the South Sea Pearl Oyster Health Management Manual is also underway. This particular project received strong support from molluscan pathology scientists from Australia, Canada, France, Japan, New Zealand and the Secretariat of the Pacific Commission who offered to assist in writing the specific chapters that will provide information on diseases and health strategies on pearl oysters in their countries.

Shrimp Disease Control and Coastal Management with India's Marine Products and Export Development Authority (MPEDA)

This project is on-going, comes in three phases and involves the following studies and activities: (a) horizontal and vertical transmission of diseases in the selected shrimp fanning areas, including investigation of hatcheries and broodstock; (b) development of practical measures for containing/preventing shrimp disease outbreaks, which should specifically cover identification of shrimp disease risk factors, diagnosis of problems and management strategies to control disease in farms; (c) conducting training and demonstration of appropriate shrimp disease control measures, which should especially include demonstration of efficient farm management practices for containing viral and other diseases in selected farms; and (d) examining opportunities for co-operation and self-help among shrimp farmers in affected areas to control water quality deterioration and shrimp disease control.

ACIAR's Surveillance Toolbox for Aquatic Animal Diseases

NACA was involved in a small group of experts organized by ACIAR which met in Bangkok, Thailand in May 2000 to develop this surveillance toolbox for aquatic animal diseases. The document is almost completed and after publication, NACA shall assist in organizing a regional workshop to introduce the concepts contained in the toolbox for pilot testing and adoption.

MRC's Mekong Basin Health Management Program

The program is currently under development and shall involve the four riparian countries of the Mekong Basin (*i.e.*, Cambodia, Lao PDR, Thailand and Vietnam). A scooping workshop is

being planned in 2002 to develop the proposal which will consider policy issues, capacity building and mechanisms for practical implementation of the 'Technical Guidelines' within the Mekong Basin.

Capacity Building, Harmonization and Inter-calibration for DNA-based Diagnostic Technologies for Detection of Prawn Viruses in the Asian Region

This project is still under development and builds on the results of ACIAR Project FIS 96/98 *Diagnostic Tests and Epidemiological Probes for Prawn Viruses in Thailand and Australia* - a cooperative project between CSIRO, Mahidol University, Thai Department of Fisheries and NACA, implemented from 1998 to 2000. The proposal will be submitted to the International Agricultural Cooperation Program of Australia with AFFA, CSIRO, Mahidol University and NACA as collaborators. The project will involve 12 NACA member governments and has four major components: (a) polymerase chain reaction training/workshop, (b) inter-government consultation, (c) inter-laboratory calibration of test sensitivities; and (d) establishment of an Asia-Pacific Prawn Health Network.

Support to other Regional/International Initiatives on Health

NACA continues to provide technical assistance to FAO's initiatives. Examples are participation in FAO TCP/RLA/0071 "Assistance to Health Management in Shrimp Aquaculture in Latin America" which is continuing and involvement of NACA to the forthcoming FAO/OIE/ DFO-Canada Expert Consultation on Surveillance and Disease Zoning being organized in Rome in October 2002.

NACA also participated in the ASEAN-SEAFDEC Millenium Conference on "Fish for the People" held in Bangkok, Thailand in November 2001 and the SEAFDEC-OIE Seminar/ Workshop on Disease Control in Fish and Shrimp Aquaculture in Southeast Asia - Diagnosis and Husbandry Techniques, held in Iloilo, Philippines in December 2001.

CONCLUSION

NACA will continue to develop projects and seek cooperation from its partners, promote enhanced and strengthened cooperation between organizations in order to provide more assistance to countries, both at national level and regional levels in our quest for resolutions to aquatic animal diseases facing our region.

NACA extends its appreciation to SEAFDEC for the invitation and support to participate at the SEAFDEC-OIE Seminar/Workshop on Disease Control in Fish and Shrimp Aquaculture in Southeast Asia - Diagnosis and Husbandry Techniques, held in Iloilo, Philippines in December 2001, to present NACA's program on aquatic animal health management.

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WORKSHOP RESULTS
AND RECOMMENDATIONS
LIST OF PARTICIPANTS
AND OBSERVERS

Workshop Results and Recommendations

During the workshop, the participants discussed the proposed training course under the Project on "Diagnosis of Shrimp and Fish Viral Diseases." This will be a trainor's training and will be conducted in the latter part of 2002. This will be different from AQD's regular training course on fish health management. The proposed training will also harmonize with the OIE disease diagnosis standards.

The following criteria were used as a guide in identifying or prioritizing the diseases that will be included in the training:

1. classified as OIE's notifiable and "significant" (*i.e.* iridovirus) diseases;
2. socio-economic importance in the region;
3. restricted geographical range and are not prevailing or difficult to be excluded;
4. no known therapeutic and husbandry measurement to control; and
5. could be diagnosed by more than one method.

The following criteria were used as guide in the selection of diagnostic methods to be used in the training:

1. should principally follow OIE diagnostic manual and standards;
2. should be practical, with chemicals and other tools needed can be sourced within the region; and
3. should give reproducible results.

Taking into consideration the above criteria and the result of the discussions, the viral diseases and their diagnostic methods that will be considered in the training course are presented in Tables 1 and 2.

Other recommendations:

1. There is a need to improve diagnostic laboratory facilities in the region.
2. There is a need to develop a standardized diagnostic kit based on OIE laboratory. The kit should also contain reference slides for each disease.
3. Reference laboratories should be made available.
4. Participants to this trainor's training should be directly involved in diagnostic work.

Table 1. Viral diseases of shrimps

Shrimp Virus	Available Diagnostic Method	Limitation
WSSV[1]	PCR[8]	
	histopathology including dark field microscopy	
	in situ and dot-blot hybridization	acceptability in the region
YHV[2]	RT-PCR[9]	availability of positive control
	histopathology	
	Western blot	availability of antibody
HPV[3]	PCR	
	histopathology	
	Giemsa-stained hepatopancreas impression smears	
MBV[4]	PCR	
	malachite green-stained hepatopancreas impression smears	
	histopathology	
IHHNV[5]	PCR	
	in situ hybridization	acceptability in the region
	dot-blot hybridization	
	histopathology	
TSV[6]	RT-PCR	availability of positive control
	in situ hybridization	acceptability in the region
	histopathology	
	antibody-based method	availability of antibody
BP[7]	wet mounts	
	histopathology	

[1] White spot syndrome virus
[2] Yellow head virus
[3] Hepatopancreatic parvovirus
[4] Monodon baculovirus
[5] Infectious hematopoietic hypodermal necrosis virus
[6] Taura syndrome virus
[7] Baculovirus penaei
[8] Polymerase chain reaction
[9] Reverse transcription-PCR

Table 2. Viral diseases of fish

Marine Fish Virus	Available Diagnostic Method	Limitation
VNN[1]	RT-PCR	
	histopathology	
	immunofluorescence (IF) or enzyme linked immunosorbent assay (ELISA)	availability of antibody and fluorescence microscope
	cell culture method using SSNN[2]-1 or GF[3]-1	supply of cell lines
Iridovirus	IF with monoclonal antibody (MAb) (tissue imprinting)	availability of positive control availability of MAb and fluorescence microscope
	PCR	

[1] Viral nervous necrosis
[2] Striped snakehead
[3] Grouper fin

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THE SOUTHEAST ASIAN FISHERIES DEVELOPMENT CENTER (SEAFDEC)

The Southeast Asian Fisheries Development Center (SEAFDEC) is a regional treaty organization established in December 1967 to promote fisheries developmment in the region. Its member countries are Japan, Malaysia, the Philippines, Singapore, Thailand, Brunei Darussalam, Socialist Republic of Vietnam, Union of Myanmar and Indonesia. Representing the Member Countries is the Council of Directors, the policy-making body of SEAFDEC. The chief administrator is the Secretary-General whose office, the Secretariat, is based in Bangkok, Thailand.

Created to develop fishery potentials in the region in response to the global food crises, SEAFDEC undertakes research on appropriate fishery technologies, trains fisheries and aquaculture technicians and disseminates fisheries and aquaculture information. To pursue these objectives, the Center established four key Departments:

1. The Training Department (TD) in Samut Prakan, Thailand, established in 1967 for marine fisheries training;
2. The Marine Fisheries Research Department (MFRD) in Singapore, established in 1967 for fishery post-harvest technology;
3. The Aquaculture Department (AQD) in Tigbauan, Iloilo, Philippines, established in 1973 for aquaculture research and development; and
4. The Marine Fishery Resources Development and Management Department (MFRDMD) in Kuala Terengganu, Malaysia, established in 1992 for the development and management of the marine fishery resources in the exclusive economic zones (EEZs) of SEAFDEC Member Countries.

OFFICE INTERNATIONAL DES EPIZOOTIES (OIE)

The International Office of Epizootics, from the French name *Office International des Epizooties* (OIE), is the world organization for animal health, which was created in 1924. In April 1993, the number of Member Countries totalled 129. The main objectives of the OIE are:

1. To promote and co-ordinate experimental or other research work concerning the causes or control of contagious diseases of livestock for which international collaboration is deemed desirable;
2. To collect and bring to the attention of Governments and their animal health services, all facts and documents of general interest concerning the course of epizootic diseases and the means used to control them; and
3. To examine international draft agreements regarding animal disease control regulations and to provide signatory governments with the means of supervising their enforcement.

The fields of interest of the OIE thus include matters of public health importance linked to the consumption of animal products (including products of aquatic animals), such as therapy and drug residues.