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).

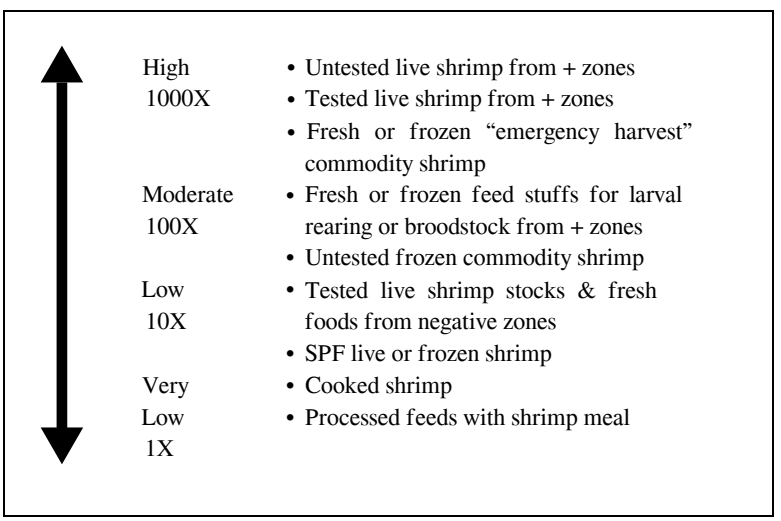

Figure 1. The relative risk presented by certain types of shrimp products, ranging from live shrimp for aquaculture, frozen shrimp for reprocessing and marketing, to processed feeds that contain shrimp meals, to the shrimp farming industry in a geographic region, a country, or a region within a country,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

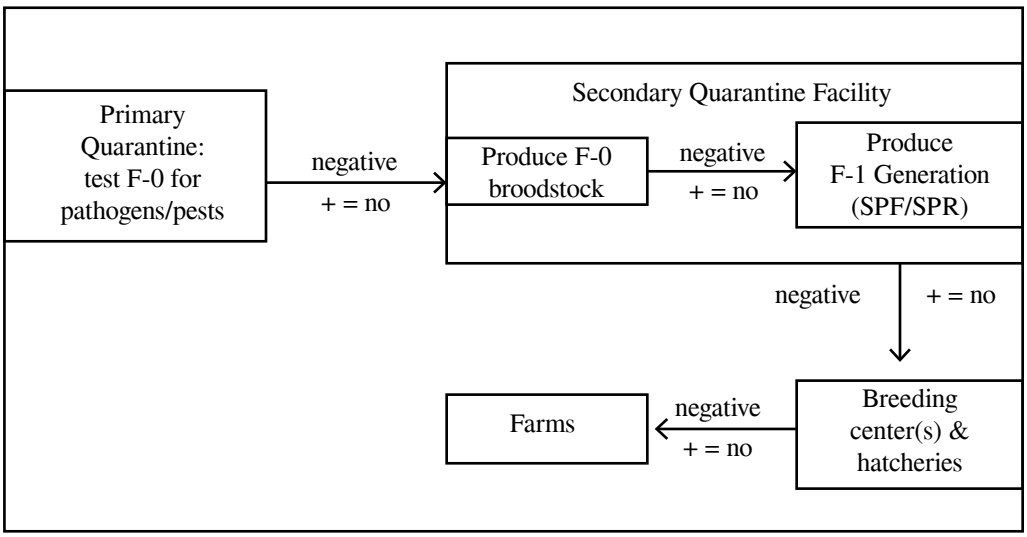

Figure 2. Schematic diagram of the steps followed by the U.S. Marine Shrimp Farming Consortium (USMSFC) in developing specific pathogen-free (SPF) breeding lines

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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