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Asia Diagnostic Guide to Aquatic Animal Diseases

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NETWORK OF
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CENTRES
IN ASIA-PACIFIC

Food
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Edited by

Melba G. Bondad-Reantaso
NACA, Bangkok, Thailand
(E-mail: Melba.Reantaso@enaca.org)

Sharon E. McGladdery
DFO-Canada, Moncton, New Brunswick
(E-mail: McGladderyS@dfo-mpo.gc.ca)

Iain East
AFFA, Canberra, Australia
(E-mail: Iain.East@affa.gov.au)

and

Rohana P. Subasinghe
FAO, Rome
(E-mail: Rohana.Subasinghe@fao.org)

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PREPARATION OF THIS DOCUMENT

The **Asia Diagnostic Guide to Aquatic Animal Diseases or 'A*sia Diagnostic Guide'*** is a comprehensive, up-datable diagnostic guide in support of the implementation of the **Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals or '*Technical Guidelines'.*** It was developed from technical contributions of members of the Regional Working Group (RWG) and Technical Support Services (TSS) and other aquatic animal health scientists in the Asia-Pacific region and outside who supported the Asia-Pacific Regional Aquatic Animal Health Management Programme. The *Asia Diagnostic Guide* is a third of a series of FAO Fisheries Technical Papers developed as part of an **FAO Technical Co-operation Project – Assistance for the Responsible Movement of Live Aquatic Animals** – implemented by NACA, in collaboration with OIE and several other national and regional agencies and organizations. The *Technical Guidelines* and the associated Beijing Consensus and Implementation Strategy (BCIS) was published as first (FAO Fisheries Technical Paper 402) of the series. The **Manual of Procedures for the Implementation of the Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals or '*Manual of Procedures'***, which provides background material and detailed technical procedures to assist countries and territories in the Asia-Pacific region in implementing the *Technical Guidelines* was the second of the series (FAO Fisheries Technical Paper 402, Supplement 1). The *Asia Diagnostic Guide* (FAO Fisheries Technical Paper 402, Supplement 2) is published as the third document of the series. All of the above-mentioned documents, developed in a highly consultative process over a period of three years (1998-2001) of consensus building and awareness raising, are in concordance with the **OIE International Aquatic Animal Code (Third Edition)** and the **OIE Diagnostic Manual for Aquatic Animal Diseases (Third Edition)** and the **WTO's Sanitary and Phytosanitary Agreement (SPS)** and in support of relevant provisions of **FAO's Code of Conduct for Responsible Fisheries (CCRF)**.

Distribution

Aquatic animal health personnel
FAO Fishery Regional and Sub-Regional Officers
FAO Fisheries Department
NACA

Cover page: Representation of relationship between host, pathogen and the environment in disease development.

Bondad-Reantaso, M.G., McGladdery, S.E., East, I., and Subasinghe, R.P. (eds.)
Asia Diagnostic Guide to Aquatic Animal Diseases.
FAO Fisheries Technical Paper No. 402, Supplement 2. Rome, FAO. 2001. 240 p.

ABSTRACT

The Asia Diagnostic Guide to Aquatic Animal Diseases or '*Asia Diagnostic Guide*' is a comprehensive, up-datable diagnostic guide for the pathogens and diseases listed in the NACA/FAO/OIE Quarterly Aquatic Animal Disease Reporting System including a number of other diseases which are significant in the Asia region. It was developed from technical contributions of members of the Regional Working Group (RWG) and Technical Support Services (TSS) and other aquatic animal health scientists in the Asia-Pacific region who supported the Asia-PacificRegional Aquatic Animal Health Management Programme. The objective was to produce an Asia diagnostic guide, that could be of specific use in the region, for both farm and laboratory level diagnostics, to complement the Manual of Procedures for the implementation of the "Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals". This Asia Diagnostic Guide could then be used to expand national and regional aquatic animal health diagnostic capabilities that will assist countries in upgrading technical capacities to meet the requirements in the OIE International Aquatic Animal Code (Third Edition) and the OIE Diagnostic Manual for Aquatic Animal Diseases (Third Edition) and WTO's Sanitary and Phytosanitary Agreement (SPS), and in support of relevant provisions in the FAO's Code of Conduct for Responsible Fisheries. The information in the *Asia Diagnostic Guide* is presented in a format that spans from gross observations at the pond or farm site (Level 1), to guidance for information on technologically advanced molecular or ultrastructural diagnostics and laboratory analyses (Levels II and III, and OIE aquatic animal health standards), thus, taking into account international, regional, and national variations in disease concerns, as well as varying levels of diagnostic capability between countries of the Asia-Pacific region.

(Key Words: Asia, Aquaculture, Diagnostics, Health Management, Aquatic Animal Diseases, Guidelines, Disease Reporting)

PREFACE

The Food and Agriculture Organization of the United Nations (FAO) and the Network of Aquaculture Centres in Asia-Pacific (NACA) are pleased to present this document entitled *Asia Diagnostic Guide to Aquatic Animal Diseases or 'Asia Diagnostic Guide'.* The *Asia Diagnostic Guide* is the third and last of a series of FAO Fisheries Technical Papers (FAO Fish. Tech. Pap. No. 402 and 402 Supplement 1), which was developed by representatives from 21 Asian governments, scientists and experts on aquatic animal health, as well as by representatives from several national, regional and international agencies and organizations. The *Asia Diagnostic Guide* provides valuable diagnostic guidance for implementing the *Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals* and their associated implementation plan, the *Beijing Consensus and Implementation Strategy (BCIS*) (see FAO Fish. Tech. Pap. No. 402). It also complements the *Manual of Procedures* for implementing the *Technical Guidelines* (see FAO Fish. Tech. Pap. No. 402, Supplement 1). The entire series is meant for assisting national and regional efforts in reducing the risks of diseases due to trans-boundary movement (introduction and transfer) of live aquatic animals. The implementation of the *Technical Guidelines* will contribute to securing and increasing income of aquaculturists in Asia by minimizing the disease risks associated with trans-boundary movement of aquatic animal pathogens. In many countries in Asia, aquaculture and capture fisheries provide a mainstay of rural food security and livelihoods, and effective implementation of the *Technical Guidelines* will contribute to regional efforts to improve rural livelihoods, within the broader framework of responsible management, environmental sustainability and protection of aquatic biodiversity.

An FAO Technical Co-operation Programme (TCP) Project (TCP/RAS 6714 (A) and 9065 (A) - "Assistance for the Responsible Movement of Live Aquatic Animals") was launched by NACA in 1998, with the participation of 21 countries from throughout the region. This program complemented FAO's efforts in assisting member countries to implement the relevant provisions in Article 9 - Aquaculture Development - of the *Code of Conduct for Responsible Fisheries* (CCRF), at both the national and regional levels. A set of Guiding Principles, formulated by a group of aquatic animal health experts at the Regional Workshop held in 1996 in Bangkok, formed the basis for an extensive consultative process, between 1998-2000, involving input from government-designated National Co-ordinators (NCs), NACA, FAO, OIE, and regional and international specialists. Based on reports from these workshops, as well as inter-sessional activities co-ordinated by FAO and NACA, the final *Technical Guidelines* were presented and discussed at the Final Project Workshop on Asia Regional Health Management for the Responsible Trans-boundary Movement of Live Aquatic Animals, held in Beijing, China, 27th-30th June 2000.

The *Technical Guidelines* were reviewed and discussed by the participants of this meeting, which included the NCs, FAO, NACA, OIE (Representatives of the Fish Disease Commission and Regional Representation in Tokyo), and many regional and international aquatic animal health management specialists. The NCs gave unanimous agreement and endorsement of the *Technical Guidelines,* in principle, as providing valuable guidance for national and regional efforts in reducing the risks of disease due to the trans-boundary movement of live aquatic animals.

Recognizing the crucial importance of implementation of the *Technical Guidelines*, the participants prepared a detailed implementation strategy, the *Beijing Consensus and Implementation Strategy* (BCIS), focussing on National Strategies and with support through regional and international co-operation. This comprehensive implementation strategy was unanimously adopted by the workshop participants.

The countries that participated in the development of the *Technical Guidelines* and *BCIS,* and the associated *Manual of Procedures* and *Asia Diagnostic Guide* are Australia, Bangladesh, Cambodia, China P.R., Hong Kong China, India, Indonesia, Iran, Japan, Korea (D.P.R.), Korea (R.O.), Lao (P.D.R.), Malaysia, Myanmar, Nepal, Pakistan, the Philippines, Singapore, Sri Lanka, Thailand and Vietnam.

PREFACE

FAO and NACA extend special thanks to all the governments, agencies, and organizations that took part in this significant, and sometimes daunting endeavor, as well as to all the individuals who generously contributed time, effort and expertise to the compilation of this document and other information produced during the process.

Ichiro Nomura
Assistant Director General
Fisheries Department
Food and Agriculture Organization of the United Nations
Viale delle Terme di Caracalla
00100 Rome, Italy
Fax: + 39 06 570-53020
E-mail: ichiro.nomura@fao.org or fi-enquires@fao.org
Website: http://www.fao.org/fi/default.asp

Pedro Bueno
Co-ordinator
Network of Aquaculture Centres in Asia-Pacific (NACA)
Department of Fisheries,
Kasetsart University Campus, Ladyao, Jatujak
Bangkok 10900, Thailand
Fax: (662) 561-1727
E-mail: Pedro.Bueno@enaca.org
Website: http://www.enaca.org

FOREWORD

Movement of live aquatic animals is a necessity for development of aquaculture on both subsistence and commercial levels. However, such movements increase the probability of introducing new pathogens, which can have dire consequences on aquaculture, capture fisheries and related resources, as well as the livelihoods which depend on them. In order to minimize or avoid the risk of pathogen transfer via aquatic animal movements, it is essential that the individuals and organizations involved in such activities appreciate, and participate in, the overall health management process.

The adverse social, economic and environmental impacts that have resulted from the irresponsible or ill-considered movement of live aquatic animals and their products have led to global recognition of the need for health management protocols to protect aquaculture, fisheries resources and the aquatic environment. In many cases, these impacts have been a direct result of the absence of effective national and regional health management strategies. However, formulation of effective quarantine measures, health certification and guidelines applicable on an international scale is complicated. A wide range of social, economic and environmental circumstances have to be considered, along with the range of aquatic animal species involved and their pathogens and diseases. In addition, differing reasons for moving live aquatic animals and products impose a further set of variables to the process. Nevertheless, the serious impacts of unrestricted regional and international movement of aquatic animals merit international recognition - a fact clearly reflected in the *International Aquatic Animal Health Code* and the *Diagnostic Manual of Aquatic Animal Diseases* of the Office International des Épizooties[1] , which provide guidelines and recommendations for reducing the risk of spreading specific pathogens considered relevant to international trade of aquatic animals.

Since present international protocols are not always applicable to the disease concerns of aquatic food production and trade in the Asia Region, the need for effective health management protocols that focus on the species and disease problems of this region has been recognized for many years. A regional, as opposed to national, approach is considered appropriate, since many countries in the region share social, economic, industrial, environmental, biological and geographical characteristics. Many countries also share waterbodies with neighbours and the watersheds of several major Asian rivers transcend national boundaries. A regionally adopted health management program will facilitate trade, and protect aquatic production (subsistence and commercial) and the environment upon which they depend, from preventable disease incursions.

A joint FAO/NACA Asia-Regional Programme on Aquatic Animal Health Management was undertaken to review the need for better health management to support safe movement of live aquatic animals and the applicability of existing international codes on aquatic animal health management, quarantine and health certification, including those of the OIE, the European Inland Fisheries Advisory Commission (EIFAC), and the International Council for Exploration of the Sea (ICES) to Asian circumstances. This review[2] highlighted the fact that the disease risks associated with pathogen transfer in the Asia Region can only be reduced through a broader approach to aquatic animal health management than currently outlined in disease-specific codes of practice (*e.g.*, the OIE code) or in codes and protocols developed specifically for northern hemisphere countries (*e.g.*, the ICES and EIFAC codes). In addition, it underlined the need for pre-border (exporter), border and post-border (importer) involvement in the program, to ensure co-operative health management of aquatic animal movement. With the support of an FAO Technical Co-operation Programme (TCP) implemented by NACA, the *Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals* is a document that was compiled by a group of aquatic animal health experts within and outside the region to assist the development of effective health management procedures for safe movement of live aquatic animals within and between countries in the region. The first companion document, the *Manual of Procedures for the Implementation of the Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals,* provides background material and detailed technical procedures to assist countries and territories in the

[1] see OIE. 2000a. International Aquatic Animal Health Code. 3rd edn. Office International des Epizooties, Paris, 153 p.; and OIE. 2000b. Diagnostic Manual for Aquatic Animal Diseases. 3rd edn, Office International des Epizooties, Paris, 237 p.

[2] see Humphrey, J.D., J.R. Arthur, R.P. Subasinghe and M.J. Phillips. 1997. Aquatic Animal Quarantine and Health Certification in Asia. Proceedings of the Regional Workshop on Health and Quarantine Guidelines for the Responsible Movement (Introduction and Transfer of Aquatic Organisms), Bangkok Thailand, 28 January 1996. FAO Fish. Techn. Pap. No. 373, 153 p.

FOREWORD

Asia Region in implementing the *Technical Guidelines*. This second companion document, Asia Diagnostic Guide, provides valuable diagnostic guidance for implementing the *Technical Guidelines* and also complementary to the *Manual of Procedures*.

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[3] The contact addresses and e-mail of persons listed are indicated elsewhere in the Asia Diagnostic Guide.

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The Editors

TABLE OF CONTENTS

Title Page ii
Disclaimer and Copyright Statements iii
Preparation of This Document iv
Abstract v
Preface vi
Foreword viii
Acknowledgements x
Table of Contents 13
Glossary 17
Abbreviations 33
Scientific and Common Names 35

SECTION 1- INTRODUCTION

I.	**INTRODUCTION**	39
I.1	**Background**	40
I.2	**Objectives and Scope**	40
I.3	**Guide for Users**	40
I.4	**Health and Aquatic Animals**	42
I.5	**Role of Diagnostics in Aquatic Animal Health**	43
I.6	**Levels of Diagnostics**	43
I.7	**References**	46

Basic Anatomy of a Typical Bony Fish 48

SECTION 2 - FINFISH DISEASES

F.1	**GENERAL TECHNIQUES**	50
F.1.1	**Gross Observations**	50
F.1.1.1	*Behaviour*	50
F.1.1.2	*Surface Observations*	50
F.1.1.2.1	Skin and Fins	50
F.1.1.2.2	Gills	51
F.1.1.2.3	Body	52
F.1.1.3	*Internal Observations*	52
F.1.1.3.1	Body Cavity and Muscle	52
F.1.1.3.2	Organs	52
F.1.2	**Environmental Parameters**	53
F.1.3	**General Procedures**	53
F.1.3.1	*Pre-Collection Preparation*	53
F.1.3.2	*Background Information*	54
F.1.3.3	*Sample Collection for Health Surveillance*	54
F.1.3.4	*Sample Collection for Disease Diagnosis*	54
F.1.3.5	*Live Specimen Collection for Shipping*	55
F.1.3.6	*Dead or Tissue Specimen Collection for Shipping*	55
F.1.3.7	*Preservation of Tissue Samples*	56
F.1.3.8	*Shipping Preserved Samples*	56
F.1.4	**Record-Keeping**	57
F.1.4.1	*Gross Observations*	57
F.1.4.2	*Environmental Observations*	57
F.1.4.3	*Stocking Records*	57
F.1.5	**References**	57

TABLE OF CONTENTS

VIRAL DISEASES OF FINFISH

F.2	**Epizootic Haematopoietic Necrosis (EHN)**	59
F.3	**Infectious Haematopoietic Necrosis (IHN)**	62
F.4	***Oncorhynchus masou Virus (OMV)***	65
F.5	**Infectious Pancreatic Necrosis (IPN)**	68
F.6	**Viral Encephalopathy and Retinopathy (VER)**	72
F.7	**Spring Viraemia of Carp (SVC)**	76
F.8	**Viral Haemorrhagic Septicaemia (VHS)**	79
F.9	**Lymphocystis**	82

BACTERIAL DISEASE OF FINFISH

F.10	**Bacterial Kidney Disease (BKD)**	86

FUNGUS ASSOCIATED DISEASE FINFISH

F.11	**Epizootic Ulcerative Syndrome (EUS)**	90

ANNEXES

F.AI	**OIE Reference Laboratories for Finfish Diseases**	95
F.AII	**List of Regional Resource Experts for Finfish Diseases in Asia-Pacific**	98
F.AIII	**List of Useful Diagnostic Manuals/Guides to Finfish Diseases in Asia-Pacific**	105

Basic Anatomy of an Oyster 108

SECTION 3 - MOLLUSCAN DISEASES

M.1	**GENERAL TECHNIQUES**	110
M.1.1	**Gross Observations**	111
M.1.1.1	*Behaviour*	111
M.1.1.2	*Shell Surface Observations*	111
M.1.1.3	*Inner Shell Observations*	111
M.1.1.4	*Soft-Tissue Surfaces*	114
M.1.2	**Environmental Parameters**	114
M.1.3	**General Procedures**	116
M.1.3.1	*Pre-Collection Preparation*	116
M.1.3.2	*Background Information*	116
M.1.3.3	*Sample Collection for Health Surveillance*	116
M.1.3.4	*Sample Collection for Disease Diagnosis*	116
M.1.3.5	*Live Specimen Collection for Shipping*	116
M.1.3.6	*Preservation of Tissue Samples*	117
M.1.3.7	*Shipping Preserved Samples*	118
M.1.4	**Record Keeping**	118
M.1.4.1	*Gross Observations*	118
M.1.4.2	*Environmental Observations*	119
M.1.4.3	*Stocking Records*	119
M.1.5	**References**	119

DISEASES OF MOLLUSCS

M.2	**Bonamiosis (*Bonamia sp., B. ostreae*)**	121
M.3	**Marteiliosis (*Marteilia refringens, M. sydneyi*)**	125
M.4	**Mikrocytosis (*Mikrocytos mackini, M. roughleyi*)**	129

TABLE OF CONTENTS

M.5	**Perkinsosis (*Perkinsus marinus, P. olseni*)**	133
M.6	**Haplosporidiosis (*Haplosporidium costale, H. nelsoni*)**	138
M.7	**Marteilioidosis (*Marteilioides chungmuensis, M. branchialis*)**	144
M.8	**Iridovirosis (*Oyster Velar Virus Disease*)**	147
	ANNEXES	
M.AI	**OIE Reference Laboratories for Molluscan Diseases**	149
M.AII	**List of Regional Resource Experts for Molluscan Diseases in Asia-Pacific**	150
M.AIII	**List of Useful Diagnostic Guides/Manuals to Molluscan Health**	152
Internal and External Anatomy of a Penaeid Shrimp		156
SECTION 4 - CRUSTACEAN DISEASES		
C.1	**GENERAL TECHNIQUES**	157
C.1.1	**Gross Observations**	157
C.1.1.1	*Behaviour*	157
C.1.1.1.1	*General*	157
C.1.1.1.2	*Mortalities*	157
C.1.1.1.3	*Feeding*	158
C.1.1.2	**Surface Observations**	158
C.1.1.2.1	*Colonisation and Erosion*	158
C.1.1.2.2	*Cuticle Softening, Spots and Damage*	158
C.1.1.2.3	*Colour*	158
C.1.1.2.4	*Environmental Observations*	160
C.1.1.3	*Soft-Tissue Surfaces*	160
C.1.2	**Environmental Parameters**	160
C.1.3	**General Procedures**	160
C.1.3.1	*Pre-collection Preparation*	160
C.1.3.2	*Background Information*	162
C.1.3.3	*Sample Collection for Health Surveillance*	162
C.1.3.4	*Sample Collection for Disease Diagnosis*	162
C.1.3.5	*Live Specimen Collection for Shipping*	162
C.1.3.6	*Preservation of Tissue Samples*	164
C.1.3.7	*Shipping Preserved Samples*	165
C.1.4	**Record-Keeping**	165
C.1.4.1	*Gross Observations*	165
C.1.4.2	*Environmental Observations*	165
C.1.4.3	*Stocking Records*	166
C.1.5	**References**	166
	VIRAL DISEASES OF SHRIMP	
C.2	**Yellowhead Disease (YHD)**	167
C.3	**Infectious Hepatopancreas and Haematopoietic Necrosis (IHHN)**	173
C.4	**White Spot Disease (WSD)**	178
C.4a	**Bacterial White Spot Syndrome (BWSS)**	183

TABLE OF CONTENTS

C.5	**Baculoviral Midgut Gland Necrosis (BMN)**	186
C.6	**Gill-Associated Virus (GAV)**	189
C.7	**Spawner Mortality Syndrome ("Midcrop mortality syndrome")**	192
C.8	**Taura Syndrome (TS)**	194
C.9	**Nuclear Polyhedrosis Baculovirosis (NPD)**	201
	BACTERIAL DISEASE OF SHRIMP	
C.10	**Necrotising Hepatopancreatitis (NH)**	207
	FUNGAL DISEASE OF CRAYFISH	
C.11	**Crayfish Plague**	211
	ANNEXES	
C.AI	**OIE Reference Laboratories for Crustacean Diseases**	215
C.AII	**List of Regional Resource Experts for Crustacean Diseases in the Asia-Pacific**	216
C.AIII	**List of Useful Manuals/Guide to Crustacean Diseases in Asia-Pacific**	219

List of National Coordinators(NCs) 221
Members of Regional Working Group (RWG) and Technical Support Services (TSS) 225
List of Figures 230

GLOSSARY[1]

Abscess	an aggregation of haemocytes (blood cells) associated with necrotic (decaying) host cells. Abscesses may or may not contain debris from invasive organisms which have been killed by host defences. In advanced abscesses there is a decrease in cell definition (especially the nuclei) towards the centre of the lesion, compared to cells around the periphery. Abscesses frequently involve breakdown of epithelial linings and may be surrounded by phagocytic and/or fibrocytic haemocytes.
Abiotic factors	physical factors which affect the development/survival of an organism
Acquired immunity	defence response developed following recovery from an infection (or vaccination) to a specific infectious agent (or group of agents)
Acute	infection or clinical manifestation of disease which occurs over a short period of time (cf 'Chronic')
Adhesion	(Crustacea) binding of subcuticular tissues to the cuticle due to destruction of the cuticle by chitinolytic bacteria or fungi. This may impede moulting.
Aetiologic Agent (Etiologic)	the primary organism responsible for changes in host animal, leading to disease
Aetiology (Etiology)	the study of the cause of disease, including the factors which enhance transmission and infectivity of the aetiologic agent.
Alevins	fry of certain species of fish, particularly trout and salmonids that still have the yolk-sac attached
Anaemia	(Vertebrate) a deficiency in blood or of red blood cells
Anorexia	loss of appetite
Antennal gland	(Crustacea) excretory pores at the base of the antennae (also known as kidney gland, excretory organ and green gland)
Antibody (Ab)	a protein capable of cross-reacting with an antigen. In vertebrates, antibody is produced by lymphoid cells in response to antigens. The mechanism of antibody production in shellfish is not known.
Antigen	a substance or cell that elicits an immune reaction. An antigen may have several epitopes (surface molecules) to which antibody can bind (cf Monoclonal and Polyclonal Antibodies).
Aquatic animals	live fish, molluscs and crustaceans, including their reproductive products, fertilised eggs, embryos and juvenile stages, whether from aquaculture sites or from the wild
Aquaculture	commonly termed "fish farming", it refers more broadly to the commercial hatching and rearing of marine and freshwater aquatic animals and plants
Ascites	accumulation of serous fluid in the abdominal cavity; dropsy
Aseptic	free from infection; sterile

[1] Definitions of words with * were adopted from OIE International Aquatic Animal Health Code. 3rd Edition. 2000. All other definitions were taken from the following references: FAO/NACA (2000); Dorland's Illustrated Medical Dictionary (27th Edition); "Virology Glossary" copyright 1995 by Carlton Hogan and University of Minnesota (permission to copy and distribute granted to individuals and non-profit groups http://www.virology.net/ATVG;ossary.html); On-line Medical Dictionary at http://www.graylab.ac.uk/omd/index.html.

GLOSSARY

Atrophy	decrease in amount of tissue, or size of an organ, after normal growth has been achieved
Autolysis(-lytic)	enzyme induced rupture of cell membranes, either as a normal function of cell replacement or due to infection
Avirulent	an infection which causes negligible or no pathology (cf Virulent).
Axenic culture	culture containing cells of a single species (bacterial culture) or cell-type (tissue culture) (uncontaminated or purified)
Bacteriology	science that deals with the study of bacteria
Bacteriophage	(abbreviation - Phage) any virus that infects bacteria
Bacterium	(bacteria) unicellular prokaryotic (nuclear material not contained within a nucleus) microorganisms that multiply by cell division (fission), typically have a cell wall; may be aerobic or anaerobic, motile or non-motile, free-living, saprophytic or pathogenic
Basophilic	acidic cell and tissue components staining readily with basic dyes (*i.e.* hematoxylin); chromatin and some secretory products in stained cells appear blue to purple
Bioassay	a quantitative procedure that uses susceptible organisms to detect toxic substances or pathogens.
Broodstock*	sexually mature fish, molluscs or crustaceans
Calcareous	pertaining to or containing lime or calcium
Cannibalism	the eating of a species of animal by the same species of animal
Carrier	an individual who harbors the specific organisms of a disease without manifest symptoms and is capable of transmitting the infection; the condition of such an individual is referred to as *carrier state*
Ceroid	non-staining metabolic by-product found in many bivalves. Abnormally high concentrations indicate possible environmental or pathogen-induced physiological stress.
Chelating agent	chemical agent used to decalcify calcium carbonate in mollusc shells or pearls, *e.g.*, ethylenediaminetetracetic acid (EDTA)
Chemotherapeutant	chemical used to treat an infection or non-infectious disorder
Chitin	linear polysaccharide in the exoskeletons of arthropods, cell walls of most fungi and the cyst walls of ciliates
Chitinolytic (chitinoclastic)	(Mycology and Bacteriology) chitin degrading organisms with enzymes capable of breaking down the chitin component of arthropod exoskeletons
Chronic	long-term infection which may or may not manifest clinical signs
Clinical	pertaining to or founded on actual observation
Chromatin	nucleoprotein complex containing genomic DNA and RNA in the nucleus of most eukaryotic cells

GLOSSARY

Chromatophores	motile, pigment-containing epidermal cells responsible for colour
Ciliostatic	exotoxin toxin secreted by some bacteria that inhibits ciliary functions
Clone	a population derived from a single organism
Coagulation	clotting (adhesion of haemocytes)
Conchiolin	nitrogenous albuminoid substance, dark brown in colour, that forms the organic base of molluscan shells
Concretions	non-staining inclusions in the tubule and kidney cells of scallops and pearl oysters, produced during the digestive cycle. Similar inclusions are also found in the gut epithelia of other bivalves.
Contagious	a disease normally transmitted only by direct contact between infected and uninfected organisms
Crustaceans*	aquatic animals belonging to the phylum Arthropoda, a large class of aquatic animals characterized by their chitinous exoskeleton and jointed appendages, *e.g.* crabs, lobsters, crayfish, shrimps, prawns, isopods, ostracods and amphipods
Cuticle	(Crustacea) the protein structure of arthropods consisting of an outer layer (epicuticle), an underlying exocuticle (pigmented), endocuticle (calcified) and membranous uncalcified layer. Chitin is in all layers except the epicuticle.
Cyst	(a) a resilient dormant stage of a free-living or parasitic organism, or (b) a host-response walling off a tissue irritant or infection
Cytology	the study of cells, their origin, structure, function and pathology
Cytopathic effect	pertaining to or characterized by pathological changes in cells
Decalcification	the process of removing calcareous matter
Decapitation	cutting of the head portion
Deoxyribovirus	(DNA-virus) virus with a deoxyribonucleic acid genome (cf Ribovirus)
DFAT	Direct Fluorescent Antibody Test/Technique; an immunoassay technique using antibody labelled to indicate binding to a specific antigen
Diapedesis	migration of haemocytes across any epithelium to remove metabolic by-product, dead cells and microbial infections
Disease	any deviation from or interruption of the normal structure or function of any part, organ, or system (or combination thereof) of the body that is manifested by a characteristic set of symptoms and signs and whose aetiology, pathology and prognosis may be known or unknown
Disease agent	an organism that causes or contributes to the development of a disease
Diagnosis*	determination of the nature of a disease
Disinfection*	the application, after thorough cleansing, of procedures intended to destroy the infectious or parasitic agents of diseases of aquatic animals; this applies to aquaculture establishments (*i.e.* hatcheries, fish farms,

	objects that may have been directly or indirectly contaminated
DNA (ssDNA, dsDNA)	deoxyribonucelic acid. Nucleic acid comprised of deoxyribonucleotides containing the bases adenine, guanine, cytosine and thymine. Single strand DNA (ssDNA) occurs in some viruses (usually as a closed circle). In eukaryotes and many viruses, DNA is double-stranded (dsDNA).
DNA probes	segments of DNA labelled to indicate detection of homologous segments of DNA in samples of tissues or cultures (see RNA probes)
Dropsy	the abnormal accumulation of serous fluid in the cellular tissues or in a body cavity
Ecdysal gland	(Crustacea) see Y-organ
Ectoparasite	a parasite that lives on the outside of the body of the host
ELISA	Enzyme Linked Immunosorbent Assay, used to detect antigen (antigen capture ELISA) or antibody (antibody capture ELISA)
Emaciation	a wasted condition of the body
Endemic	present or usually prevalent in a population or geographical area at all times
Endothelial	pertaining to or made up of endothelium
Endothelium	the layer of epithelial cells that lines the cavities of the heart and of the blood and lymph vessels, and the serous cavities of the body originating from the mesoderm
Endosymbiosis	an association between two organisms (one living within the other) where both derive benefit or suffer no obvious adverse effect
Envelope	(Virology) lipoprotein membrane composed of host lipids and viral proteins (non-enveloped viruses are composed solely of the capsid and nucleoprotein core)
Enzootic	present in a population at all times but, occurring only in small numbers of cases
Eosinophilic	basic cell and tissue components staining readily with acidic dyes (*i.e.* eosin); stained cells appear pink to red
Epibiont	organisms (bacteria, fungi, algae, *etc.*) which live on the surfaces (cf fouling) of other living organisms
Epipodite	(Crustacea) cuticular extension of the base (protopodite) of the walking legs (pereiopods)
Epitope	the component of an antigen which stimulates an immune response and which binds with antibody
Epizootic	affecting many animals within a given are at the same time; widely diffused and rapidly spreading (syn. Epidemic - used for human disease)
Epidemiology	science concerned with the study of the factors determining and influenc ing the frequency and distribution of disease or other health related events

	and their causes in a defined population for the purpose of establishing programs to prevent and control their development and spread
Epizootiology	the study of factors influencing infection by a pathogenic agent
Epithelium	the layer of cells covering the surface of the body and all gastrointestinal linings. Epithelia are usually one cell thick and supported by a basal membrane.
Epitope	structural component of an antigen which stimulates an immune response and which binds with antibody.
Erosion	destruction of the surface of a tissue, material or structure
Eukaryotean	organism that contains the chromosones within a membrane-bound nucleus (cf Prokaryote)
Exoenzyme	extracellular enzyme released by a cell or microorganism
Exopthalmia	abnormal protrusion of the eyeballs
Exoskeleton	(Crustacea) the chitin and calcified outer covering of crustaceans (and other arthropods) which protects the soft-inner tissues
Exudate	material, such as fluid, cells, or cellular debris, which has escaped from blood vessels and has been deposited in tissues or on tissue surfaces, usually as a result of inflammation
Euthanasia	an easy or painless death
Filtration	passage of a liquid through a filter, accomplished by gravity, pressure or vacuum (suction)
Finfish*	fresh or saltwater fish of any age
Fry	newly hatched fish larvae
Fingerling	a young or small fish
Fixation	preservation of tissues in a liquid that prevents protein and lipid breakdown and necrosis; the specimen is hardened to withstand further processing; and the cellular and sub-cellular contents are preserved in a manner close to that of the living state
Fixative	a fluid (*e.g.* aldehyde or ethanol-based solutions)) that prevents denatur ation and autolysis by cross-linking of proteins
Foreign bodies	any organism or abiotic particle not formed from host tissue
Formalin	a 37% solution of formaldehyde gas
Fouling	the mass colonisation of hard substrates by free-living organisms. Extreme fouling of living organisms, such as molluscs or shrimp, can impede their normal body-functions leading to weakening and death
Fungus	any member of the Kingdom Fungi, comprising single-celled or multinucle ate organisms that live by decomposing and absorbing the organic material in which they grow oyster farms, shrimp farms, nurseries), vehicles, and different equipment/

GLOSSARY

Gaping	weakened molluscs that cannot close their shells when removed from water; this rapidly lead to desiccation or predation of the soft-tissues and is indicative of molluscs in poor condition (including possible infection)
Gram's Stain	stain used to differentiate bacteria with permeable cells walls (Gram-negative) and less permeable cell walls (Gram-positive)
Granulomas	any small nodular delimited aggregation of granular haemocytes, or modified macrophages resembling epithelial cells (epithelioid cells)
Granulomatosis	any condition characterized by the formation of multiple granulomas
Granulosis virus	Baculoviridae belonging to subgroup (B), characterised by a single nucleocapsid within an envelope. Granulosis viruses form intra-nuclear ellipsoid or rounded occlusion bodies (granules or capsules) containing one or two virions.
Gross signs	signs of disease visible to the naked eye
Haematopoietic	pertaining to or effecting the formation of blood cells
Haematopoietic tissue	(Decapoda) a sheet of tissue composed of small lobules surrounded by fibrous connective tissue which lies along the dorso-lateral surfaces of the posterior portion of the cardiac stomach (Brachyura) or surrounding the lateral arterial vessels, secondary maxillipeds and epigastric tissues (Penaeidae and Nephropidae); (Bivalves) unknown; (Vertebrates) spleen
Haemocytes	blood-cells
Haemolymph	cell-free fraction of the blood containing a solution of protein and non-proteinaceous defensive molecules
Haemocyte infiltration	accumulation of haemocytes around damaged or infected tissues; since the type of haemocytes most commonly responsible for phagocytosis are granulocytes, focal infiltration is often referred to as a "granuloma"
Haemocytopenia	a reduction in the number of cells in the circulatory system, usually associated with a reduction in blood-clotting capability
Haemocytosis	systemic destruction of blood cells (syn. Haemolysis)
Haemorrhage	(Vertebrate) escape of blood from the vessels; bleeding (Invertebrate) uncontrolled loss of haemocytes due to tissue trauma, epithelial rupture, chronic diapedesis
Hatcheries*	aquaculture establishments raising aquatic animals from fertilized eggs
Hepatopancreas	digestive organ composed of ciliated ducts and blind-ending tubules, which secrete digestive enzymes for uptake across the digestive tubule epithelium; also responsible for release of metabolic by-products and other molecular or microbial wastes (cf Metaplasia, Diapedesis)
Histology	the study that deals with the minute structure, composition and function of tissues
Histolysis	breakdown of tissue by disintegration of the plasma membranes
Histopathology	structural and functional changes in tissues and organs of the body which

	cause or are caused by a disease seen in samples processed by histology
Homogenate	tissue ground into a liquid state in which all cell structure is disinte grated
Host	individual organism infected by another organism
Husbandry	management of captive animals to enhance reproduction, growth and health
Hyperplasia	abnormal increase in size of a tissue or organ due to an increase in number of cells
Hypertrophy	abnormal enlargement of cells due to irritation or infection by an intracellu lar organism.
Hyphae	(Mycology) tubular cells of filamentous fungi; may be divided by cross-walls (septae) into multicellular hyphae, may be branched. Inter-connecting hyphae are called mycelia.
Icosahedral	shape of viruses with a 5-3-2 symmetry and 20, approximately equilateral, triangular faces
IFAT	Indirect Fluorescent Antibody Test/Technique; a technique using unlabelled antibody and a labelled anti-immunoglobulin to form a 'sandwich' with any antigen-bound antibody
Immunity	protection against infectious disease conferred either by the immune response generated by immunization or previous infection or by other non-immunologic factors
Immunization	protection against disease by deliberate exposure to pathogen antigens to induce defence system recognition and enhance subse quent responses to exposure to the same antigens (syn Vaccination)
Immunoassay	any technique using the antigen-antibody reaction to detect and quantify the antigens, antibodies or related substances (see ELISA, IFAT, DFAT)
Immunodepression	decrease in immune system response to antigens due to an infection (same or different agent) or exposure to an immunosupressant chemical.(syn. Immunosupression)
Immunofluorescence	any immuno-histochemical method using antibody labeled with a fluorescent dye
	Direct - if a specific antibody or antiserum with a fluorochrome and used as a specific fluorescent stain
	Indirect - if the fluorochrome is attached to an antiglobulin, and a tissue constituent is stained using an unlabeled specific antibody and the labeled antiglobulin, which binds the unlabeled antibody
Immunoglobulin (Ig)	family of proteins constructed of light and heavy molecular weight chains linked by disulphide bonds; usually produced in response to antigenic stimulation
Immunohistochemistry	application of antigen-antibody interactions to histochemical tech

	niques, as in the use of immunofluorescence
Immunology	branch of biomedical science concerned with the response of the organisms to antigenic challenge, the recognition of self and not self, and all the biological (*in vivo*), serological (*in vitro*), and physical chemical aspects of immune phenomena
Immunostimulation	enhancement of defense responses, *e.g.*, with vaccination
Immunization	induction of immunity
Inclusion body	non-specific discrete bodies found within the cytoplasm or nucleus of a cell. Frequently viral (cf Cowdry body, Polyhedrin Inclusion /Occlusion Bodies), or bacterial microcolonies (cf RLOs) (syn. Inclusions)
Infectious	capable of being transmitted or of causing infection
Infection	invasion and multiplication of an infectious organism within host tissues. May be clinically benign (cf sub-clinical or 'carrier') or result in cell or tissue damage. The infection may remain localized, subclinical, and temporary if the host defensive mechanisms are effective or it may spread an acute, sub-acute or chronic clinical infection (disease).
Infiltration	(Invertebrates) haemocyte migration to a site of tissue damage or infection by a foreign body/organism ('inflammation'). Infiltration may also occur for routine absorption and transport of nutrients and disposal of waste products.
Inflammation	(Vertebrate) initial response to tissue injury characterised by the release of amines which cause vasodilation, infiltration of blood cells, proteins and redness that may be associated with heat generation
	(Invertebrates) infiltration response to tissue damage or a foreign body. The infiltration may be focal, diffuse or systemic (syn. Infiltration).
Innate immunity	host defence mechanism that does not require prior exposure to the pathogen
Intensity of infection	the number of infectious agents in an individual organism or specimen; "mean" intensity is the average number of infectious agents present in all infected individuals in a sample
Intercellular	situated or occurring between the cells in a tissue
Interstitial tissue	tissue or cells between epithelial bound organ systems; also known as (cells) Leydig tissue (molluscs) or connective tissue
Intracellular	situated or occurring within a cell
Intrapallial	(Bivalves) space between the mantle, gills and other soft-tissues; the space between the mantle and inner shell is the extrapallial space
Karyolysis	a form of necrosis where the chromatin leaches out of the nucleus without disrupting the nuclear membrane, leaving an 'empty' appearing nucleus
Karyorrhexic	rupture of the nucleus and nuclear membrane, releasing chromatin granules into the cytoplasm
Lesion	any pathological or traumatic change in tissue form or function

GLOSSARY

Lethargy	abnormal drowsiness or stupor (response only to vigorous stimulation); a condition of indifference
Liquefaction	conversion of a tissue into a semi-solid or fluid mass due to necrosis
Luminescent	marine or euryhaline bacteria which contain luciferase (a fluorescent, bacteriaenzyme) *e.g., Vibrio harveyi* and *V. splendidus*
Lymphoid organ	(Crustacea) an organ situated between the anterior and posterior stomach chambers which connects the sub-gastric artery to the anterior aorta, via a mass of interconnected tubules
Lymphoid organ spheres	spherical cellular masses composed of presumed phagocytic haemocytes, which sequester Taura Syndrome Virus (TSV) and aggregate within intertubular spaces of the lymphoid organs
Macrophages	(Vertebrates) large (10-20 mm) amoeboid blood cells, responsible for ph agocytosis, inflammation, antibody and cytotoxin production.
Mandibular organ	(Crustacea) large glandular organ close to the ventral epidermis between the mandibles; believed to be related to the moulting cycle, although it does not produce a known moult-inducing hormone
Mantle retraction/ recession	during periods of no growth in molluscs, the mantle retracts away from the edge of the shell. Prolonged mantle retraction leaves the inner shell edge open to erosion and fouling.
Melanin	dark brown-black polymer (pigment) of indole quinone which has enzyme inhibiting properties. It forms part of the primary defence mechanism against cuticle and epidermal damage in many crustaceans
Melanisation	abnormal deposits of dark pigment in various organs or tissues
Melanophores	(Crustacea) dermal cells containing melanin (syn. melanocytes)
Metaplasia	the change in shape of any epithelial cell, *e.g.*, from columnar to cuboidal or squamous (flattened)
Microcolonies	membrane-bound populations of Chlamydia bacteria or non-membrane bound Rickettsial colonies (cf Inclusion bodies)
Microorganism	principally, viruses, bacteria and fungi (microscopic species, and taxo nomically-related macroscopic species). Microscopic protistans (Protozoa) and algae may also be referred to as microorganisms.
Molecular probes	see DNA probes
Molluscs*	aquatic organism belonging to the Phylum Mollusca in the Kingdom Metazoa characterized by soft unsegmented bodies. Most forms are enclosed in a calcareous shell. The different developmental stages of molluscs are termed larvae, postlarvae, spat, juvenile and adult.
Monoclonal antibody (MaB)	identical antibody molecules produced by clonage of the antibody producing cell and responsive to a single antigen epitope (cf Epitope)
Moribund	diseased; near death
Mortality	death

Moulting	(Crustacea) the shedding of the exoskeleton to permit growth (increase in size) of internal soft-tissues (syn. Ecdysis)
Mucous	pertaining or relating to, or resembling mucus
Mucus	the free slime of the mucous membrane, composed of secretion of the glands, along with various inorganic salts, desquamated cells and leukocytes
Multiple aetiology	disease associated with more than one infectious agent; may be directly attributed to one or more infectious organism (cf Syndrome)
Mycelial colonies	(Bacteriology) colony growth of Gram-positive Actinomycete bacteria with branched mycelia which may fragment into rods or coccoid forms
Mycelium	(Mycology) network formed by interconnecting hyphae (syn. Mycelial network)
Mycology	the study of fungi (Mycota)
Mycosis	any disease resulting from infection by a fungus
Myodegeneration	breakdown of muscle fibres
Mysis larvae	(Crustacea) pelagic larval stage between protozoea (zoeal) and post larva
Nacre	inner layer of molluscan shells; may have an iridescent crystal matrix (mother-of-pearl)
Nauplius(-plii)	(Crustacea) earliest larval stage; with three pairs of appendages, uniramous first antennae, biramous second antennae and mandibles
Necrosis	sum of the morphological changes indicative of cell death and caused by the progressive and irreversible degradative action of enzymes; it may affect groups of cells or part of a structure or an organ; necrosis may take different forms and be associated with saprobionts (bacterial, fungal or protistan) proliferation.
Notifiable Diseases*	'diseases notifiable to the OIE' means the list of transmissible diseases that are considered to be of socio-economic and/or public health impor tance within countries and that are significant in the international trade in aquatic animals and aquatic animal products (see also OIE 1997, OIE 2000a, b)
Nuclear Polyhedrosis Virus (NPV)	Baculoviruses (Type A) which produce intranuclear polyhedral protein matrices (see Polyhedral Occlusion/Inclusion Bodies)
Nucleocapsid	protein-nucleic acid complex which may form the core, capsid and/or helical nucleoprotein of the virion
Occlusion	(vascular) filling or blocking of vascular sinuses by haemocytes; (perivascu lar) infiltration of haemocytes, several cells deep into the tissues surround ing vascular sinuses; (luminal) filling or blocking of gonoducts, renal ducts, digestive tubules or ducts by haemocytes or other cell debris
Occlusion body	(see Polyhedrin Inclusion/Occlusion Body)

GLOSSARY

Oedema (edema)	presence of abnormally large amounts of fluid in the intercellular spaces of the body
Opportunistic	organism capable of causing disease only when a host's resistance is pathogen lowered by other factors (another disease, adverse growing conditions, drugs, *etc.*)
Osmoregulation	maintenance of osmolarity by a simple organism or body cell with respect to the surrounding medium
Other Significant Diseases*	diseases that are of current or potential international significance in aquaculture, but that have not been included in the list of diseases notifiable to the OIE because they are less important than the 'notifiable diseases', or because their geographical distribution is limited, or is too wide for notification to be meaningful,or it is not yet sufficiently defined, or because the aetiology of the disease is not well enough understood, ar approved diagnostic methods are not available (see also OIE 1997, OIE 2000a, b)
Outbreak	the sudden onset of disease in epizootic proportions
Overt	open to view; not concealed
Parasite	an organism which lives upon or within another living organism (host) at whose expense it obtains some advantage, generally nourishment
Parasitology	science that deals with the study of parasites
Passage	(Virology) the successive transfer of a virus or other infectious agent through a series of experimental animals, tissue culture, or synthetic media with growth occurring in each medium
Patent infection	period when clinical signs and/or the infectious organism can be detected (cf Prepatent)
Pathogen	an infectious agent capable of causing disease
Pathogenicity	the ability to produce pathologic changes or disease
Pathognomonic	sign or symptom that is distinctive for a specific disease or pathologic condition
Pathology	deals with the essential nature of disease, especially of the structural and functional changes in tissues and organs of the body which cause or are caused by a disease
PCR	Polymerase Chain Reaction, a process by which nucleic acid sequences can be replicated ('nucleic acid amplification')
Pereiopods	(Crustacea) thoracic appendages ('walking legs') (cf Pleopods and Uropods)
Periostracum	(Molluscs) calcareous layers of shell which may contain quinine-tanned protein
Phages	(see Bacteriophage)
Phagocytosis	uptake by a cell of material from the environment by invagination of its plasma membrane

GLOSSARY

Plasma membrane	trilaminar membrane enclosing the cytoplasm and organelles of a cell
Pleiopod	small legs of some crustaceans
Pleomorphic	organism demonstrating more than one body form within a life-cycle
Polyadenalated RNA	messenger RNA (mRNA) which has a polyadenylate sequence bound to the 3' end of the molecule. This is common in most eukaryote mRNA and is present in some riboviruses. The function of this addition is unknown.
Polyclonal antibodies (PAb)	(more correctly, but rarely, termed 'Polyclonal antiserum') an antiserum prepared from an organism exposed to an antigen. The PAb contains several different antibodies, each specific to a different epitope of the same antigen. (see Monoclonal antibody).
Polyhedral Inclusion/ Occlusion Body (POB, PIB)	protein-based crystalline matrix made up of Polyhedrin (Baculovirus group A - Nuclear Polyhedrosis Viruses (NPV)) or Granulin (Baculovirus group B - Granulosis Viruses (GV)). Baculovirus group C do not form occlusion bodies.
Polymorphic	(a) capability of molecules, such as enzymes, to exist in several forms; (b) ability of nuclei of certain cells (*e.g.*, haemocytes) to change shape; and (c) ability of microorganisms to change shape (*e.g.*, in different host species or tissues)
Pop-eye	abnormal protrusion of the eyes from the eye sockets
Postlarvae (PL)	the stage following metamorphosis from larvae to juvenile in the life cycle of Crustacea. In penaeid shrimp, this is commonly counted in days after appearance of postlarval features, *e.g.*, PL12 indicates a post-larvae that has lived 12 days since its metamorphosis from the zoea stage of development.
Predator	an organism that derives elements essential for its existence from organisms of other species, which it consumes and destroys
Predispose	to make susceptible to a disease which may be activated by certain conditions, as by stress
Preening	(Crustacea) cleaning surface tissues or eggs exposed to fouling (cf Epibionts and Fouling); some crustaceans have modified appendages to enhance preening (*e.g.*, the gill-rakers of Brachyura)
Prepatent period	period between infection and the manifestation of clinical or detectable signs of disease
Prevalence	percentage of individuals in a sample infected by a specific disease, parasite or other organism
Prokaryote	(syn. Bacteria) cellular micro-organisms in which the chromsones are not enclosed within a nucleus
Prophylactic (-axis):	action or chemotherpeutant administered to healthy animals in order to prevent infection (see Treatment)
Pustule	a sub-epidermal swelling containing necrotic cell debris as a result of inflammation (haemocyte infiltration) in response to a focal infection

Putative	signifies that which is commonly thought, reputed or believed
Pyknosis/Pyknotic	contraction of nuclear contents to a deep staining (basophilic) irregular mass, sign of death cell (cf Karryorhexis and Karyolysis)
Quarantine	holding or rearing of aquatic animals under conditions which prevent their escape, and the escape of any pathogens they may be carrying, into the surrounding environment. This usually involves sterelisation/disinfection of all effluent and quarantine materials. Quarantine measures are measures developed as a result of risk analysis to prevent the transfer of disease agents with live aquatic animal move ments, with pre-border, border and post-border health management processes, however, such activities are equally applicable to intra-national movements of live aquatic animal.
Repair	process to re-establish anatomical and functional integrity of tissues after an injury or infection
Reservoir	(host or infection) an alternate or passive host or carrier that harbors pathogenic organisms, without injury to itself, and serves as a source from which other individuals can be infected
Resistance	(to Disease) (cf Acquired immunity and Innate immunity) the capacity of an organism to control the pathogenic effects of an infection. Resistance does not necessarily negate infection ('Refraction') and varying degrees of tolerance to the infection may be manifest. Heavy sub-clinical infections are indicative of resistance (syn. Tolerance; opp. Susceptible)
Resistance	(Antibiotic or 'drug' resistance) the capability of a microbe to evade destruction by an antibiotic. This may arise from changes in the antigenic properties of the microbe. Survival and multiplication leads to development of drug resistant strains of the pathogen. This may confer resistance to related (heteroresistance) or non-related antibiotics (multiple drug resistance).
Ribosomes	intracytoplasmic granules which are rich in RNA and function in protein synthesis
Ribovirus (RNA-virus)	virus with a ribonucleic acid (see RNA) genome (see Deoxyribovirus)
Risk	the probability of negative impact(s) on aquatic animal health, environmen tal biodiversity and habitat and/or socio-economic investment(s)
RNA	ribonucleic acid consisting of ribonucleotides made up of the bases (ssRNA, dsRNA) adenine, guanine, cytosine and uracil
RNA probes	segments of RNA which are labelled to detect homologous segments of RNA or DNA in tissue or culture samples (cf DNA probes)
rRNA	(Ribosomal RNA) RNA component of the ribonucleoprotein organelle responsible for protein synthesis within a cell
Saprobionts	(syn. Saprotroph) organisms which obtain nutrition from dead organic matter
Schizonts	the multinucleated stage or form of development during schizogony

GLOSSARY

Secondary	infection infection resulting from a reduction in the host's resistance as a consequence of an earlier infection
Septicaemia	systemic disease associated with the presence and persistence of pathogenic microorganisms or their toxins in the blood; blood poisoning
Serology	term now used to refer to the use of such reactions to measure serum antibody titers in infectious disease (serologic tests), to the clinical correlations of the antibody titer (the 'serology' of a disease) and the use of serologic reactions to detect antigens
Serum	fluid component of coagulated haemolymph
Shipment*	a group of aquatic animals or products thereof destined for transportation
Sporangium	(Mycology) hyphal swelling which contains motile or non-motile zoospores; release is via a pore or breakdown of the sporangial wall. (syn. Zoospo rangium)
Sporangium	(Bacteriology) the cell, or part of a cell, which subsequently develops into an endospore (intracellularly formed spore)
Spore	infective stage of an organism that is usually protected from the environ ment by one or more protective membranes (syn. Zoospores)
Sporogenesis	formation of or reproduction by spores; sporulation
Sterilization	any process (physical or chemical) which kills or destroys all contaminating organisms, irrespective of type; a sterile environment (aquatic or solid) is free of any living organism
Stress	the sum of biological reactions to any adverse stimuli (physical, internal or external) that disturb the organism's optimum operating status
Sub-clinical	(asymptomatic) an infection with no evident symptoms or clinical signs of disease, or a period of infection preceding the onset of clinical signs (cf Prepatent)
Surveillance*	a systematic series of investigations of a given population of aquatic animals to detect the occurrence of disease for control purposes, and which may involve testing of samples of a population
Susceptible	an organism which has no immunity or resistance to infection by a another organism
Syndrome	an assembly of clinical signs which when manifest together are indicative of a distinct disease or abnormality (syn. Pathognomic/ Pathognomonic)
Synergistic	(infection) pathology increased by two or more infections by different agents, compared with the effect from individual effects (opp. to 'antago nistic' or 'suppressive', where one infection counteracts the other)
Systemic	pertaining to or affecting the body as a whole
Systemic infection	an infection involving the whole body
Tail rot	disintegration of tail and fin tissue
Telson	(Crustacea) terminal segment of the abdomen which overlies the uropods

Tomont	the non-feeding, dividing stage or form in the life cycle of certain protozoa that typically encysts and produces tomites by fission
Transmission	transfer of an infectious agent from one organism to another
	Horizontal - direct from environment (*e.g.*, via ingestion, skin and gills)
	Vertical - prenatal transmission (*i.e.*, passed from parent to egg); may be either inside the egg (intra-ovum) or through external exposure to patho gens from the parent generation
Transport	movement of stocks between locations by human influence
Trauma	an effect of physical shock or injury
Treatmentaction	taken to eradicate an infection (cf Prophylaxis)
Trophozoites	the active, motile, feeding stage of a protozoan organism, as contrasted with the non-motile encysted stage
Tumour	abnormal growth as a result of uncontrolled cell division of a localised group of cells
Ubiquitous	existing or being everywhere
Ulcer	excavation of the surface of an organ or tissue, involving sloughing of necrotic inflammatory tissue.
Uropods	(Crustacea) the terminal appendages underlying the telson that form the 'tail fan' (see Pereiopods and Pleopods)
Vaccine	an antigen preparation from whole or extracted parts of an infectious organism, which is used to enhance the specific immune response of a susceptible host
Vacuolated	containing spaces or cavities within the cytoplasm of a cell
Veliger	(Mollusc) ciliated planktonic larval stage
Velum (Velar)	(Mollusc) ciliated feeding surface of veliger larvae
Viable	capable of living or causing a disease
Virion	individual viral particlecontaining nucleic acid (the nucleoid), DNA or RNA (but not both) and a protein shell, or capsid
Virogenic stroma(e)	site of viral replication or assembly (syn. Viroplasm)
Virogensis	production of virions
Virology	branch of microbiology which is concerned with the study of viruses and viral diseases
Virulence	the degree of pathogenicity caused by an infectious organism, as indicated by the severity of the disease produced and its ability to invade the tissues of the host; the competence of any infectious agent to produce pathologic effects; virulence is measured experimentally by the median lethal dose (LD_{50}) or median infective dose (ID_{50})

GLOSSARY

Virus	one of a group of minute infectious agents, characterized by a lack of independent metabolism and by the ability to replicate only within living host cells
Y-organ (Crustacea)	(syn. Ecdysal gland) gland resonsible for production of the moulting hormone ecdysone. Production of the moulting hormone is controlled by a moult inhibiting hormone synthesised in the eye-stalk
Zoea larvae	(Crustacea) stage following metamorphosis from the nauplius larva, characterised by four pairs of thoracic appendages; may be referred to as protozoea where differentiation between the nauplius and mysis or postlarva stage of development is difficult
Zoospores	motile, flagellated and asexual spores

ABBREVIATIONS

BF-2	Bluegill-Fin 2
BKD	Bacterial kidney disease
BMN	Baculoviral Midgut Gland Necrosis
BMNV	Baculoviral Midgut Gland Necrosis Virus
BP	*Baculovirus penaei*
BWSS	Bacterial white spot syndrome
CAIs	Cowdry type A inclusion bodies
CHSE-214	Chinook salmon embryo-214
CPE	Cytopathic effect
CSHV	Coho Salmon Herpesvirus
CSTV	Coho Salmon Tumour Virus
CTAB	cetyltrimethylammonium bromide
DFAT	Direct fluorescent antibody test
DNA	Deoxyribonucleic acid
dd	double distilled
dsDNA	double stranded DNA
DTAB	dodecyltrimethylammonium bromide
EHN	Epizootic Haematopoietic Necrosis
EHNV	Epizootic Haematopoeitic Necrosis Virus
ELISA	Enzyme-linked Immunosorbent Assay
EPC	*Epithelioma papulosum cyprinae*
ERA	EUS-related *Aphanomyces*
EUS	Epizootic Ulcerative Syndrome
FBS	fetal bovine serum
FEV	Fish Encephalitis Virus
FHM	Fathead Minnow
GAV	Gill Associated Virus
GP	glucose peptone
GPY	glucose peptone yeast
H&E	Haematoxylin & Eosin
HHNBV	Baculoviral Hypodermal and Haematopoietic Necrosis
1G4F	1% Glutaraldehyde : 4% Formaldehyde
ICTV	International Committee on Taxonomy of Viruses
IFAT	Indirect Fluorescent Antibody Test
IgG	primary antibody (IgG)
IHHN	Infectious Hypodermal and Hematopoietic Necrosis
IHHNV	Infectious Hypodermal and Hematopoietic Necrosis Virus
IHN	Infectious Haematopoietic Necrosis
IHNV	Infectious Haematopoietic Necrosis Virus
IPN	Infectious Pancreatic Necrosis
IPNV	Infectious Pancreatic Necrosis Virus
ISH	*in situ* hybridization
kDa	kilodalton
KDM2	Kidney Disease Medium
KDMC	Kidney Disease Medium Charcoal
LDV	Lymphocystis Disease Virus
LOS	‘lymphoid organ spheroids’
LOV	Lymphoid Organ Virus
LOVV	Lymphoid Organ Vacuolisation Virus
LPV	Lymphoidal Parvo-like Virus
Mab	Monoclonal antibody
MCMS	Mid-crop Mortality Syndrome
MEM	Minimal Essential Medium
MG	Mycotic Granuloma-fungus
“MSX”	multinucleate sphere X
NeVTA	Nerka virus Towada Lake, Akita and Amori prefecture
NHP	Necrotising Hepatopancreatitis
NPB	Nuclear Polyhedrosis Baculovirosis
OKV	*Oncorhynchus kisutch* virus

ABBREVIATIONS

OTC	oxytetracycline
OMV	*Oncorhynchus masou* virus
OVVD	Oyster Velar Virus Disease
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PKD	Proliferative Kidney Disease
PL	Postlarvae
PNHP	Peru Necrotizing Hepatopancreatitis
RDS	“runt deformity syndrome”
RHV	Rainbow Trout Herpesvirus
RKV	Rainbow Trout Kidney Virus
RNA	Ribonucleic Acid
RSD	Red spot disease
RTG-2	Rainbow Trout Gonad-2
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RV-PJ	Rod-shaped Nuclear Virus of *Penaeus japonicus*
RVC	*Rhabdovirus carpio*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electophoresis
SEED	Shrimp Explosive Epidemic Disease
SEMBV	Systemic Ectodermal and Mesodermal Baculovirus
SJNNV	Striped Jack Nervous Necrosis Virus
SKDM	Selective Kidney Disease Medium
SMV	Spawner-isolated Mortality Virus
SMVD	Spawner-isolated Mortality Virus Disease
SPF	Specific Pathogen Free
ssDNA	single stranded DNA
ssRNA	single stranded RNA
“SSO”	seaside organism
SSN-1	Striped Snakehead (*Channa striatus*) cell-line
SVC	Spring Viraemia of Carp
SVCV	Spring Viraemia of Carp Virus
TEM	Transmission Electron Microscopy
TNHP	Texas Necrotizing Hepatopancreatitis
TPMS	Texas Pond Mortality Syndrome
TS	Taura Syndrome
TSV	Taura Syndrome Virus
UV	ultraviolet
VER	Viral Encephalopathy and Retinopathy
VHS	Viral Haemorrhagic Septicaemia
VHSV	Viral Haemorrhagic Septicaemia Virus
VIMS	Virginia Institute of Marine Science
VNN	Viral Nervous Necrosis)
YBV	Yellowhead Baculovirus
YHD	Yellowhead disease
YHV	Yellowhead Virus
YHDBV	Yellowhead Disease Baculovirus
YHDLV	Yellow-Head-Disease-Like virus
YTV	Yamame tumour virus
WSBV	White Spot Baculovirus
WSD	White Spot Disease
WSS	White Spot Syndrome
WSSV	White Spot Syndrome Virus

SCIENTIFIC AND COMMON NAMES

A. FINFISH (Hosts)

Scientific Name	Common Name
Argentina sphyraena	lesser argentine
Aristichthys nobilis	bighead carp
Bidyanus bidyanus	silver perch
Carassius auratus	goldfish
Carassius carassius	crucian carp
Channa striatus	striped snakehead
Chanos chanos	milkfish
Clupea harengus	herring
Clupea pallasi	Pacific herring
Coregonus spp.	white fish
Ctenopharyngodon idellus	grass carp
Cyprinus carpio	common carp
Dicentrarchus labrax	European sea bass
Epinepheles akaara	red-spotted grouper
Epinephelus malabaricus	brown spotted grouper
Epinephelus moara	kelp grouper
Esox lucius	pike
Gadus macrocephalus	Pacific cod
Gadus morhua	Atlantic cod
Galaxias olidus	mountain galaxias
Gambussia affinis	mosquito fish
Hippoglossus hippoglossus	halibut
Hypophthalmichthys molitrix	silver carp
Ictalurus melas	catfish
Labroides dimidatus	doctor fish
Lates calcarifer	sea bass, Australian barramundi
Macquaria australasica	Macquarie perch
Melanogrammus aeglefinus	haddock
Merlangius merlangius	whiting
Micromesistius poutassou	blue whiting
Mugil cephalus	grey mullet
Oncorhynchus keta	chum salmon
Oncorhynchus kisutch	coho salmon
Oncorhynchus masou	sockeye salmon/Yamame salmon/masou salmon
Oncorhynchus mykiss	rainbow or steelhead trout
Oncorhynchus nerka	Kokanee (non-anadromous sockeye) salmon
Oncorhynchus rhodurus	amago salmon
Oncorhynchus tshawytscha	chinook salmon
Oplegnathus fasciatus	Japanese parrotfish
Oplegnathus punctatus	rock porgy
Oreochromis spp.	Tilapia
Paralichthys olivaceus	Japanese flounder
Perca fluviatilis	redfin perch
Plecoglossus altivelis	ayu
Poecilia reticulata	guppy
Pseudocaranx dentex	striped jack
Rhinonemus cimbrius	rockling
Salmo salar	Atlantic salmon
Salmo trutta	brown trout
Salvelinus fontinalis	brook trout
Scophthalmus maximus	turbot
Seriola quinqueradiata	Japanese yellowtail flounder
Silurus glanis	sheatfish
Sparus aurata	gilt-head sea bream

Sprattus sprattus	sprat
Takifugu rubripes	tiger puffer
Tinca tinca	tench
Thymallus thymallus	grayling
Trisopterus esmarkii	Norway pout
Umbrina cirrosa	shi drum

B. MOLLUSCS (Hosts)

Scientific Name	Common Name
Acanthogobius flavimanus	Japanese yellow goby
Arca sp.	clams
Argopecten gibbus	Calico scallop
Austrovenus stutchburyi	New Zealand cockles
Barbatia novae-zelandiae (Family Arcidae)	(not available)
Cerastoderma (= *Cardium*) *edule*	Common European cockle
Crassostrea angulata	Portuguese oysters
Crassostrea ariakensis	Ariake cupped oyster
Crassostrea commercialis	Sydney rock oyster
Crassostrea gigas	Pacific oyster
Crassostrea virginica	American oysters
Crassostrea angulata	Portugese oysters
Haliotis cyclobates	abalone
Haliotis laevigata	greenlip abalone
Haliotis roei	abalone
Haliotis rubra	blacklip abalone
Haliotis scalaris	abalone
Macomona liliana (Family Tellinidae)	(bivalve, not available)
Mercenaria mercenaria	hard shell clam
Mytilus edulis	edible mussel
Mytilus galloprovincialis	edible mussel
Ostrea angasi	flat oyster (southern mud oyster)
Ostrea conchaphila (*O. lurida*)	Olympia oyster
Ostrea edulis	European oyster
Ostrea lutaria (*Tiostrea lutaria*)	New Zealand oyster
Ostrea puelchana	(not available)
Patinopecten yessoensis	Japanese (Yesso) scallops
Pinctada albicans	pearl oyster
Pinctada maxima	Mother of pearl
Pteria penguin	winged pearl oyster
Ruditapes decussatus	European clam
Ruditapes philippinarum	Manila clam
Saccostrea commercialis	Sydney rock oyster
Saccostrea (*Crassostrea*) *cucullata*	Mangrove oyster
Saccostrea echinata	Northern black lip oyster
Saccostrea glomerata	Sydney rock oysters
Scrobicularia plana	Peppery furrow shell
Tiostrea chilensis (*Ostrea chilensis*)	South American oyster
Tiostrea lutaria	(not available)
Tridacna maxima	giant clam

C. CRUSTACEANS (Hosts)

Scientific Name	Common Name
Acetes spp. (Crustacea:Sergestidae)	krill, small shrimp
Cherax quadricarinatus	freshwater crayfish, red claw
Euphausia spp.	krill

SCIENTIFIC AND COMMON NAMES

Marsupenaeus (Penaeus) japonicus	Kuruma prawn
Metapenaeus ensis	red endeavour or greasy back shrimp/prawn
Palaemon styliferus	(not available)
Penaeus aztecus	Northern brown shrimp
Penaeus californiensis	yellowleg shrimp
Penaeus chinensis	Chinese white shrimp
Penaeus duodarum	caged pink shrimp
Penaeus esculentus	brown tiger shrimp/prawn
Penaeus indicus	Indian or red legged banana shrimp/prawn
Penaeus japonicus	Japanese king or Kuruma shrimp/prawn
Penaeus marginatus	Aloha prawn
Penaeus merguiensis	common or Gulf banana shrimp/prawn
Penaeus monodon	giant black tiger shrimp/prawn
Penaeus occidentalis	Western white shrimp
Penaeus paulensis	pink shrimp
Penaeus penicillatus	redtail prawn, beige colored shrimp
Penaeus plebejus	Eastern king shrimp/prawn
Penaeus schmitti	white shrimp
Penaeus semisulcatus	grooved tiger or green tiger shrimp/prawn
Penaeus setiferus	Native white shrimp
Penaeus stylirostris	blue shrimp
Penaeus subtilis	Southern brown shrimp
Penaeus vannamei	white shrimp

D. Pathogens/Disease Agents

Aeromonas hydrophila
Argulus foliaceus
Argulus spp
Aphanomyces astaci
Aphanomyces invadans
Aphanomyces invaderis
Aphanomyces piscicida
Baculovirus penaei
Bonamia ostreae
Bonamia sp
Dermocystidium marinum
Haplosporidium costale
Haplosporidium. Nelsoni
Herpervirus
Hexamita inflata
Hexamita salmonis
Mytilicola sp.
Labyrinthomyxa marinus
Lerneae cyprinacea
Marteilia maurini
Marteilia refringens
Marteilia sydneyi
Marteilioides branchialis
Marteilioides christenseni
Marteilioides chungmuensis
Marteilioides lengehi
Mikrocytos mackini
Mikrocytos roughleyi
Minchinia costale
Minchinia nelsoni
Myxobolus artus
Ligula sp.
Perkinsus atlanticus

Perkinsus marinus
Perkinsus olseni
Perkinsus qugwadi
Piscicola geometra
Polydora sp.
Posthodiplostomum cuticola
Ranavirus
Renibacterium salmoninarum
Rhabdovirus carpio
Salmincola salmoneus
Staphylococcus aureus
Vibrio harveyi
Vibrio splendidus
Vibrio spp

SECTION 1- INTRODUCTION

I.	**INTRODUCTION**	39
I.1	**Background**	40
I.2	**Objectives and Scope**	40
I.3	**Guide for Users**	40
I.4	**Health and Aquatic Animals**	42
I.5	**Role of Diagnostics in Aquatic Animal Health**	43
I.6	**Levels of Diagnostics**	43
I.7	**References**	46

I. INTRODUCTION

I.1 Background

The FAO Regional Technical Cooperation Programme (TCP) Project ***"Assistance for Responsible Movement of Live Aquatic Animals"*** (TCP/RAS/6714-A and 9605-A), was implemented in January 1998 by NACA , in cooperation with the OIE[1], regional and international agencies (*e.g.* AAHRI[2], AusAID/APEC[3], AFFA[4], and others), representatives (designated National Coordinators and focal points for disease reporting) of 21 governments/territories in the Asia-Pacific region (Australia, Bangladesh, Cambodia, China PR, Hong Kong SAR China, India, Indonesia, Iran, Japan, Korea (DPR), Korea (RO), Lao PDR, Malaysia, Myanmar, Nepal, Pakistan, Philippines, Singapore, Sri Lanka, Thailand and Vietnam) and many regional and international aquatic animal disease experts. The over-all objective of the program was to provide guidance to countries in undertaking responsible movement (introductions and transfers) of live aquatic animals through appropriate strategies that minimize potential health risks associated with live aquatic animal movements. The program took into account the need for concordance with existing international agreements/treaties (*e.g.* WTO's SPS Agreement and OIE health standards) along with the need for the strategies to be practically applicable to the Asia region and in support for FAO's Code of Conduct for Responsible Fisheries (CCRF). This TCP became the focal point for the development of a strong, multidisciplinary Asia-Pacific Regional Programme on Aquatic Animal Health Management which is a major element of NACA's Five Year Work Programme (2001-2005). The ***"Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals and the Beijing Consensus and Implementation Strategy (TGBCIS)"*** or ***'Technical Guidelines'*** (FAO/NACA 2000) and the corresponding ***"Manual of Procedures (MOP)"*** (FAO/NACA 2001) were developed over a period of three years (from 1998-2001) of awareness and consensus building in consultation (through various national level and regional workshops, FAO/NACA/OIE 1998) with government representatives, representatives of collaborating organizations and aquatic animal health experts. The *'Technical Guidelines'* was finally adopted in principle during a Final Workshop of the TCP held in Beijing, China PR in June 2000 (FAO/NACA 2000). The **Asia Diagnostic Guide to Aquatic Animal Diseases** or ***'Asia Diagnostic Guide'*** is a third of a series of documents produced under the TCP that will support the implementation of the ***'Technical Guidelines'*** particularly with respect to the component on disease diagnosis, surveillance and reporting.

The ***'Asia Diagnostic Guide'*** is a comprehensive diagnostic manual for the pathogens and diseases listed in the NACA/FAO/OIE Quarterly Aquatic Animal Disease Reporting System[5]. It was developed from technical contributions from members of the Regional Working Group (RWG) and Technical Support Services (TSS) of the TCP and other aquatic animal health scientists in the Asia-Pacific region and outside who supported the regional programme.

Many useful aquatic animal health diagnostic guides and manuals and others in CD-ROM format already exist in the literature. Some are in

[1] Office International des Epizooties
[2] Aquatic Animal Health Research Institute of the Thai Department of Fisheries
[3] Australian Agency for International Development/Asia-Pacific Economic Cooperation
[4] Agriculture, Fisheries and Forestry of Australia
[5] The quarterly reporting system was developed as one of the four major components of the TCP, developed based on the OIE International Aquatic Animal Health Code – 1997, in cooperation with the OIE Regional Representation for Asia and the Pacific.

INTRODUCTION

the language of individual countries. In the Asia-Pacific region, more recent ones include Indonesia's Manual for Fish Disease Diagnosis - II (Koesharyani *et al.* 2001, GRIM[6]/JICA[7]); the Philippines' Diseases of Penaeid Shrimps in the Philippines (Lavilla-Pitogo *et al.* 2000, SEAFDEC-AQD[8]); Thailand's (a) Diagnostic Procedures for Finfish Diseases (Tonguthai *et al.* 1999, AAHRI), (b) Health Management in Shrimp Ponds, Third Edition (Chanratchakool *et al.* 1998, AAHRI), and (c) Epizootic Ulcerative Syndrome (EUS) Technical Handbook (Lilley *et al.* 1998, ACIAR[9]/DFID[10]/AAHRI/NSW[11]-Fisheries/NACA); Australia's Australian Aquatic Animal Disease – Identification Field Guide (Herfort and Rawlin 1999, AFFA); and a CD-ROM on Diagnosis of Shrimp Diseases (Alday de Graindorge and Flegel 1999). Some more are listed and appear as an Annex in the different sections of the *Asia Diagnostic Guide.*

The ***'Asia Diagnostic Guide'*** supplements these existing manuals/guides and provides relevant information on diseases in the ***NACA/FAO and OIE Asia-Pacific Quarterly Aquatic Animal Disease Reporting System,*** which commenced during 3rd quarter of 1998 (NACA/FAO 1999, OIE 1999). The information in the *Asia Diagnostic Guide* is presented in a format that spans from gross observations at the pond or farm site (Level 1), to guidance for information on technologically advanced molecular or ultrastructural diagnostics and laboratory analyses (Levels II and III, and OIE 2000a, b), thus, taking into account international, regional, and national variations in disease concerns, as well as varying levels of diagnostic capability between countries of the Asia-Pacific region.

I.2 Objectives and Scope

The objective of the ***"Asia Diagnostic Guide"*** is to produce a manual/guide of specific use for both farm and laboratory level aquatic animal disease diagnostics in the Asia region that complements the *'Manual of Procedures'* and that which will serve as a supplement to the implementation of the *'Technical Guidelines'.* The *Asia Diagnostic Guide* is aimed at providing a tool that can be used to expand national and regional aquatic animal diagnostic capacities and the infrastructure required to meet the OIE aquatic animal health standards (OIE 2000a, b). This guide aims to improve aquatic animal health awareness as well as provide knowledge on how to access the diagnostic resources required to help prevent or control disease impacts.

The ***Asia Diagnostic Guide*** focuses on the NACA/FAO and OIE listed diseases, but also includes some which are significant in parts of the Asia-Pacific region.

I.3 Guide for Users

The ***Asia Diagnostic Guide*** is divided into four sections: **Section 1** on Introduction, Background, Scope and Purpose, Guide for Users, Health and Aquatic Animals, Role of Diagnostics and Levels of Diagnostics; **Sections 2 to 4**, divided into host groups, *i.e.* **Finfish Diseases (Section 2)**, **Molluscan Diseases (Section 3)** and **Crustacean Diseases (Section 4)**, each commences with a chapter on *"General Techniques"* which covers the essential "starting points" that will enable prompt and effective response(s) to disease situations in aquatic animal production. This chapter is not disease-specific, providing information applicable to a wide range of both infectious and noninfectious disease situations. It emphasizes the importance of gross observations (Level 1), and how and when they should be made. It also describes environmental parameters worth recording, general procedures for sampling and fixation and the importance of record-keeping. Each **General Techniques** section is divided as follows:

Gross Observations
- *Behaviour*
- *Surface Observations*

Environmental Parameters

General Procedures
- *Pre-collection Preparation*
- *Background Information*
- *Sample Collection for Health Screening*
- *Sample Collection for Disease Diagnosis*
- *Live Specimen Collection and Shipping*
- *Dead or Tissue Specimen Collection and Shipping*
- *Preservation of Tissues*
- *Shipping Preserved Specimens*

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[6] Gondol Research Institute for Mariculture of the Central Research Institute for Sea Exploration and Fisheries, Indonesia's Department of Marine Affairs and Fisheries
[7] Japan International Cooperation Agency
[8] Aquaculture Department of the Southeast Asian Fisheries Development Center
[9] Australian Centre for International Agricultural Research
[10] Department for International Development of the United Kingdom
[11] New South Wales (Australia)

Table I.2.1. NACA/FAO and OIE listed diseases and other diseases[12] covered in the *Asia Diagnostic Guide.*

DISEASES PREVALENT IN SOME PARTS OF THE REGION
Finfish diseases
1. Epizootic haematopoietic necrosis* (EHN)
2. Infectious haematopoietic necrosis* (IHN)
3. *Oncorhynchus masou* virus disease* (OMVD)
4. Infectious pancreatic necrosis (IPN)
5. Viral encephalopathy and retinopathy (VER)
6. Epizootic ulcerative syndrome (EUS)
7. Bacterial kidney disease (BKD)
Mollusc diseases
1. Bonamiosis * (*Bonamia* sp., *B. ostreae*)
2. Marteiliosis * (*Marteilia refringens, M. sydneyi*)
3. Microcytosis * (*Mikrocytos mackini, M. roughleyi*)
4. Perkinsosis * (*Perkinsus marinus, P. olseni*)
Crustacean diseases
1. Yellowhead disease (YHD)
2. Infectious hypodermal and haematopoietic necrosis (IHHN)
3. White spot disease (WSD)
4. Baculoviral midgut gland necrosis (BMN)
5. Gill associated virus (GAV)
6. Spawner mortality syndrome ('Midcrop mortality syndrome') (SMVD)
DISEASES PRESUMED EXOTIC TO THE REGION, BUT REPORTABLE TO THE OIE
Finfish diseases
1. Spring viraemia of carp* (SVC)
2. Viral haemorrhagic septicaemia* (VHS)
Mollusc diseases
1. Haplosporidiosis* (*Haplosporidium costale, H. nelsoni*)
OTHER DISEASES WITHIN THE REGION, NOT CURRENTLY LISTED
Finfish diseases
1. Lymphocystis
Mollusc diseases
1. Marteilioidosis (*Marteilioides chungmuensis, M. branchialis*)
2. Iridovirosis (Oyster velar virus disease)
Crustacean diseases (the following diseases are so far presumed, but not proven, to be exotic to this region)
1. Taura Syndrome (TS)
2. Nuclear Polyhedrosis Baculovirosis (*Baculovirus penaei*)
3. Necrotising hepatopancreatitis
4. Crayfish plague

*OIE Notifiable Diseases (OIE 1997)

[12] The diseases listed in the Asia-Pacific Quarterly Aquatic Animal Disease Reporting System were agreed through a process of consultation with the National Coordinators, members of the Regional Working Group (RWG) and Technical Support Services (TSS), FAO, NACA and OIE based on the OIE International Aquatic Animal Health Code – 1997, including some diseases which are deemed important to the Asia-Pacific region.

Record-keeping
Gross Observations
Environmental Observations
Stocking Records
References

The "General Techniques" section is followed, under each host group, by a number of chapters aimed at specific diseases (*e.g.* viral, bacterial, fungal) listed on the current NACA/FAO and OIE quarterly reporting list (Table 1.2.1). These are recognised of being of regional importance, as well as of international trade significance. Those diseases listed as "Notifiable" or "Other Significant Diseases" by the OIE are cross-referenced to the most up-to-date version of the OIE *Diagnostic Manual for Aquatic Animal Diseases* (OIE 2000b, also available at http://www.oie.int). The diagnostic techniques described in the *Asia Diagnostic Guide* are consistent with those recommended by OIE. Since it is recognised that disease diagnostics is a dynamic field, it is highly recommended that **anyone using this manual for the purpose of health certification for international transfers of live aquatic organisms refer to the OIE diagnostic manual prior to performing diagnostics (screening) for this purpose**. In addition, other diseases/infectious agents *not* presently included on the Regional Quarterly Reporting List are included in the *Asia Diagnostic Guide*, since they are of interest to the region and infect commercially significant species.

Each chapter on specific diseases is presented with information on the following:

- **Causative Agent(s)**- an introductory paragraph on the causative agent(s) responsible for the disease.
- **Host Range** – the range of hosts that can be infected (both naturally and experimentally).
- **Geographic Distribution** - known/recorded geographic range of the disease (updated, where applicable, using the Asia-Pacific Quarterly Aquatic Animal Disease Reports for 1999-2000 (OIE, NACA/FAO quarterly reports).
- **Clinical Aspects** - the effects of the disease are described, ranging from gross observations and behavioural changes, lesions and other external clues, to gross and microscopic internal pathology.
- **Screening Methods** – are the examination methods applied to check healthy appearing aquatic animals to determine whether or not they are infected by a potentially significant infectious agent.
- **Diagnostic Methods** – are the examination procedures used to try and determine the cause of a disease or clinical infection.

  Screening and diagnostic methods are divided into two types:

  Presumptive Diagnosis – preliminary diagnosis based on gross observations and circumstantial evidence. Where more than one infectious agent may be responsible, confirmatory diagnosis (usually laboratory Level II and/or III) may be required; and

  Confirmatory Diagnosis – positive identification of the causative agent, with a high degree of diagnostic confidence.
- **Modes of Transmission** - deals with the known modes of transmission (spread) of the disease and the factors associated with its spread (environmental, handling, life-history stage, reservoirs of infection, *etc.*). This area of diagnostics is known as epidemiology (or epizootiology) and available observations from this field of study are also included.
- **Control Measures** – describes any control measures which are known to work, should the disease appear.
- **References** – recent relevant publications about the disease.

The chapters for each host group also include **three Annexes** providing information of the (a) list of OIE Reference Laboratories, (b) a list of regional disease experts who can provide information and valuable health advise, and (c) useful guides/manuals. A **Glossary** is also included.

I.4 Health and Aquatic Animals

Unlike other farm and harvesting situations, where the animals and plants are visible, aquatic animals require more attention in order to monitor their health. They are not readily visible, except under tank-holding conditions, and they live in a complex and dynamic environment. Likewise, feed consumption and mortalities may be equally well hidden under water. Unlike the livestock sector, aquaculture has a wide range of diversity in species cultured, farming environment, nature of containment, intensity of practice and culture system used. The range of diseases found in aquaculture is also varied, some with low or unknown host specificities and many with non-specific symptoms. Disease is now recognized as one of the most important challenges facing the aquaculture sector.

The complexity of the aquatic ecosystem makes the distinction between health, sub-optimal performance and disease obscure. Diseases in aquaculture are not caused by a single

event but are the end result of a series of linked events involving the interactions between the host (including physiological, reproductive and developmental stage conditions), the environment and the presence of a pathogen (Snieszko 1974). Under aquaculture conditions, three factors are particularly important affecting host's susceptibility: stocking density, innate susceptibility and immunity (natural/acquired). Environment includes not only the water and its components (such as oxygen, pH, temperature, toxins, wastes) but also the kind of management practices (*e.g.* handling, drug treatments, transport procedures, *etc.*). Pathogens may include viruses, bacteria, parasites and fungi; diseases may be caused by a single species or a mixture of different pathogens. The introduction of infectious diseases is another major concern in aquaculture. As in livestock, the aquaculture and fisheries sector will continue to face increasing global exposure to disease agents as it intensifies trade in live aquatic organisms and their products (Subasinghe *et al.* 2001).

The first and most important defenses against preventable disease losses under such complex situations are:

monitoring *as regularly as possible* and *appropriate* action at the *first* sign(s) of suspicious behaviour, lesions or mortalities.

These fundamental approaches – despite having been long instilled in human and agricultural production – still require reinforcement in many aquatic animal production sectors. Some farmers and harvesters still hesitate to act at the first sign of health problems, due to concern that it may reflect on their production capability, or that it will result in failure in the competitive market place. Hiding or denying health problems, however, can be as destructive to aquatic animals as it is elsewhere. It is important to recognise that disease is a challenge that everyone has to face, and having the resources that can effectively deal with it, are the primary weapons against misplaced ignorance and fear.

I.5 Role of Diagnostics in Aquatic Animal Health Management and Disease Control

Diagnostics play two significant roles in aquatic animal health management and disease control. As described above, some diagnostic techniques are used to screen healthy animals to ensure that they are not carrying infection at sub-clinical levels by specific pathogens. This is most commonly conducted on stocks or populations of aquatic animals destined for live transfer from one area or country to another. Such screening provides protection on two fronts: (a) it reduces the risk that animals are carrying few, if any, opportunistic agents which might proliferate during shipping, handling or change of environment; and (b) it reduces the risk of resistant or tolerant animals transferring a significant pathogen to a population which may be susceptible to infection. The second role of diagnostics is to ***determine the cause of unfavourable health or other abnormality*** (such as spawning failure, growth or behaviour) in order to recommend mitigating measures applicable to the particular condition. This is the most immediate, and clearly recognised, role of diagnostics in aquatic animal health.

Accurate diagnosis of a disease is often incorrectly described as complicated and costly. This may be the case for some of the more difficult to diagnose diseases or newly emerging diseases. Disease diagnosis is ***not solely a laboratory test***. A laboratory test may confirm the presence of a specific disease agent, or it may exclude its presence with a certain level of certainty. Incorrect diagnosis can lead to ineffective or inappropriate control measures (which may be even more costly). For example, a "new" disease agent may get introduced to a major aquaculture producing area, or the animals may all die in shipment/during handling. Disease diagnostics should be made as a continuum of observations starting on the farm and, in fact, commencing prior to the disease event. The different levels of disease diagnostics which can be undertaken when investigating a disease situation are discussed in the section below.

I.6 Levels of Diagnostics

The *Asia Diagnostic Guide* is built on a framework of ***"three levels" of diagnostics***, agreed upon during the Second Regional Workshop of the TCP held in Bangkok in February 1999 (see FAO/NACA 2000). Table I.6.1 below outlines the diagnostic activities at each level, who is responsible, and the equipment and training required. It should be noted that none of the levels function in isolation, but build on each other, each contributing valuable data and information for optimum diagnoses. Level 1 provides the foundation and is the basis of Levels II and III since findings using higher level(s) can only be meaningfully interpreted only in conjunction with observations and results obtained from lower levels.

Level I (farm/production site observations, record-keeping and health management) is strongly emphasized throughout the *Asia Diagnostic Guide* as this forms the basis for triggering the other diagnostic levels (II and III). ***Level II*** includes the specialisations of parasitology, histopathology, bacteriology and mycology, which require moderate capital and training investment, and which, generally-speaking, cannot be conducted at the farm or culture site. ***Level III*** comprises the types of advanced diagnostic specialisation which requires significant capital and training investment. As the reader will note, immunology and biomolecular techniques are included in Level III, although field kits are now being developed for farm or pond-side use (Level I) as well as use in microbiology or histology laboratories (Level II). These efforts are good indication that technology transfer is now enhancing diagnostics and, with solid quality control and field validation, it is certain that more Level III technology will become field accessible in the near future (Walker and Subasinghe 2000).

One of the most important aspects of maximising the effectiveness of the three diagnostic levels is ensuring that Level I diagnosticians have access to, and know how to contact Levels II and III support (and at what cost), and *vice versa*. Level III diagnostic support is usually based on referrals, so has little contact with field growing conditions. They, therefore, need feedback to ensure any diagnosis (and actions recommended) are relevant to aquatic animal production situation being investigated.

Thus, the baseline aim for initiating diagnostic capability is Level I. Confirmatory diagnostics, or second opinion, *where required*, can be obtained by referral until such capabilities are developed locally. The period required to develop Level II and/or Level III diagnostic infrastructures, usually depends on the disease situations being faced and tackled by Level I diagnosticians in the area/country and the resources available. Where there are few problems, there is little incentive to build diagnostic capability. This is a vulnerable position, and strong links with Level II and/or III diagnostics are good precautionary measures and strongly promoted under the regional program – especially for introductions of live aquatic animals into a relatively disease-free area.

Table I.6.1 Diagnostic Levels, Associated Requirements and Responsibilities.

Level	Activity	Work requirements	Responsibility	Technical requirements to support activities
I	Observation of animal and environment Gross clinical examination	Knowledge of normal (feeding, behaviour, growth) of stock. Frequent / regular observation of stock. Regular, consistent record-keeping and assistance (Levels II, III). maintenance of records – including fundamental environmental information. Knowledge contacts for health diagnosis 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. Eqiopment lists Model clinical observation sheets. Pond/Site record sheets. Preservation/transportation guidelines for Levels II/IIIdiagnoses. Model job descriptions/skill requirements. *Asia Diagnostic Guide for Aquatic Animal Diseases*
II	Parasitology Bacteriology Mycology Histopathology	Laboratories with basic equipment and personnel trained/experienced in aquatic animal pathology. Keep and maintain accurate diagnostic and laboratory case records. Ability to preserve and storage specimens for optimal Level III diagnoses. Knowledge of/ contact with different areas of specialisation within Level II. Knowledge of who to contact for Level III diagnostic assistance.	Fish biologists/ technicians. Aquatic Veterinarians. Parasitologists/ technicians. Mycologists/ technicians Bacteriologists/ 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/ skill lists Access to Level II and Level III specialist expertise *Asia Diagnostic Guide for Aquatic Animal Diseases* *OIE Diagnostic Manual for Aquatic Animal Diseases* Regional General Diagnostics Manuals
III	Virology Electron microscopy Molecular biology Immunology	Highly equipped laboratory with highly specialised and trained personnel. Keep and maintain accurate diagnostic and laboratory case records. Preserve and store specimens. Maintenance of contact with people responsible for sample submission.	Virologist/ technician. Ultrastructural histopatholo-gist/ technicians. Molecular biology scientists/ technicians.	Model laboratory requirements/ equipment/ consumables lists Model job descriptions/ skill requirements Contact information for reference laboratories Protocols for preservation of samples for consultation/ validation *Asia Diagnostic Guide for Aquatic Animal Diseases* *OIE Diagnostic Manual for Aquatic Animal Diseases* General molecular and microbiology diagnostic references

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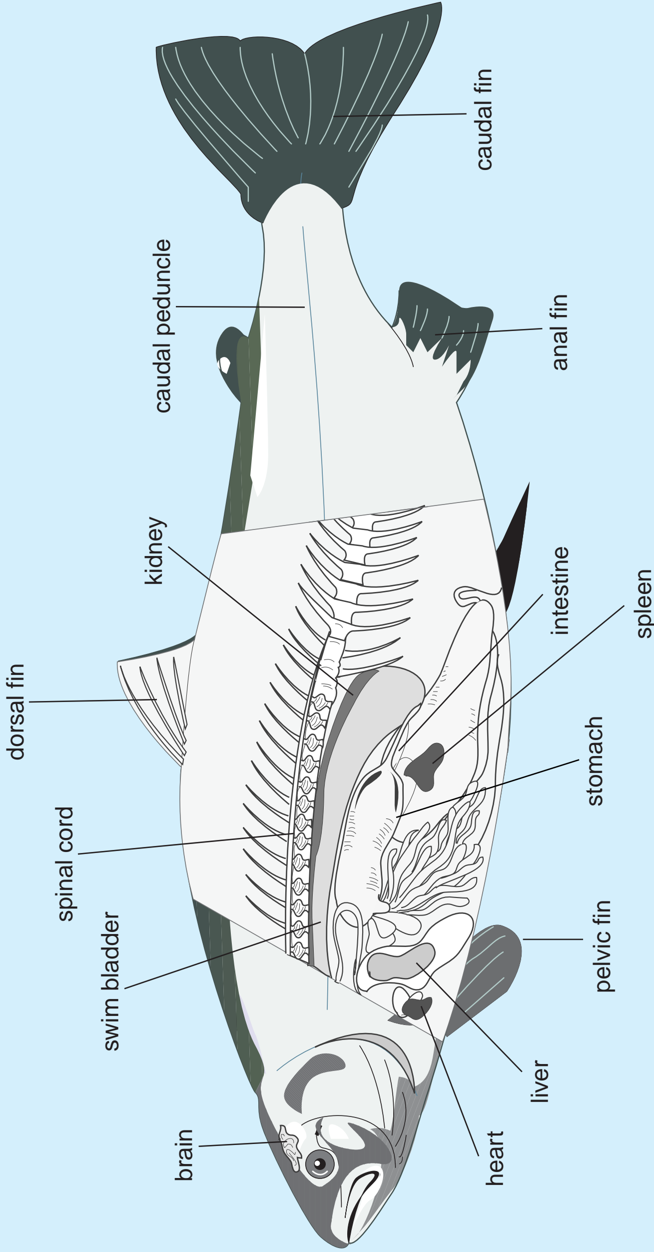
Basic Anatomy of a Typical Bony Fish



Basic anatomy of a typical bony fish.

SECTION 2 - FINFISH DISEASES

Basic Anatomy of a Typical Bony Fish		48
SECTION 2 - FINFISH DISEASES		
F.1	**GENERAL TECHNIQUES**	50
F.1.1	**Gross Observations**	50
F.1.1.1	*Behaviour*	50
F.1.1.2	*Surface Observations*	50
F.1.1.2.1	Skin and Fins	50
F.1.1.2.2	Gills	51
F.1.1.2.3	Body	52
F.1.1.3	*Internal Observations*	52
F.1.1.3.1	Body Cavity and Muscle	52
F.1.1.3.2	Organs	52
F.1.2	**Environmental Parameters**	53
F.1.3	**General Procedures**	53
F.1.3.1	*Pre-Collection Preparation*	53
F.1.3.2	*Background Information*	54
F.1.3.3	*Sample Collection for Health Surveillance*	54
F.1.3.4	*Sample Collection for Disease Diagnosis*	54
F.1.3.5	*Live Specimen Collection for Shipping*	55
F.1.3.6	*Dead or Tissue Specimen Collection for Shipping*	55
F.1.3.7	*Preservation of Tissue Samples*	56
F.1.3.8	*Shipping Preserved Samples*	56
F.1.4	**Record-Keeping**	57
F.1.4.1	*Gross Observations*	57
F.1.4.2	*Environmental Observations*	57
F.1.4.3	*Stocking Records*	57
F.1.5	**References**	57
	VIRAL DISEASES OF FINFISH	
F.2	**Epizootic Haematopoietic Necrosis (EHN)**	59
F.3	**Infectious Haematopoietic Necrosis (IHN)**	62
F.4	***Oncorhynchus masou Virus (OMV)***	65
F.5	**Infectious Pancreatic Necrosis (IPN)**	68
F.6	**Viral Encephalopathy and Retinopathy (VER)**	72
F.7	**Spring Viraemia of Carp (SVC)**	76
F.8	**Viral Haemorrhagic Septicaemia (VHS)**	79
F.9	**Lymphocystis**	82
	BACTERIAL DISEASE OF FINFISH	
F.10	**Bacterial Kidney Disease (BKD)**	86
	FUNGUS ASSOCIATED DISEASE FINFISH	
F.11	**Epizootic Ulcerative Syndrome (EUS)**	90
	ANNEXES	
F.AI	**OIE Reference Laboratories for Finfish Diseases**	95
F.AII	**List of Regional Resource Experts for Finfish Diseases in Asia-Pacific**	98
F.AIII	**List of Useful Diagnostic Manuals/Guides to Finfish Diseases in Asia-Pacific**	105

F.1 GENERAL TECHNIQUES

General fish health advice and other valuable information are available from the OIE Reference Laboratories, Regional Resource Experts in the Asia-Pacific, FAO and NACA. A list is provided in Annexes F.AI and AII, and up-to-date contact information may be obtained from the NACA Secretariat in Bangkok (e-mail: naca@enaca.org). Other useful guides to diagnostic procedures which provide valuable references for regional parasites, pests and diseases are listed in Annex F.AIII.

F.1.1 **Gross Observations**

F.1.1.1 *Behaviour* (Level I)

At a time when there are no problems on the farm, "normal behaviour" of the animals should be observed to establish and describe the "normal" situation. Any change from normal behaviour should be a cause for concern and warrants investigation. Prior to the clinical expression of disease signs, individual finfish may exhibit increased feed consumption followed by cessation of feeding, or the fish may simply go off feed alone. Taking note of normal feed conversion ratios, length/weight ratios or other body-shape signs described below, is essential in order to detect impending disease.

Abnormal behaviour includes fish swimming near the surface, sinking to the bottom, loss of balance, flashing, cork-screwing or air gulping (non air-breathers) or any sign which deviates from normal behaviour. Bursts of abnormal activity are often associated with a generalised lethargy. Behavioural changes often occur when a fish is under stress. Oxygen deprivation leads to gulping, listlessness, belly-up or rolling motion. This can be due to blood or gill impairment. Flashing can indicate surface irritation, *e.g.*, superficial secondary infections of surface lesions. Corkscrew and other bizarre behaviour may also indicate neurological problems that may be disease related (see F.6 - Viral Encephalopathy and Retinopathy).

Patterns of mortalities should be closely monitored, as well as levels of mortality. If losses persist or increase, samples should be sent for laboratory analysis (Level II and/or III). Mortalities that seem to have a uniform or random distribution should be examined immediately and environmental factors during, pre- and post-mortality recorded. Mortalities that spread from one area to another may suggest the presence of an infectious disease agent and should be sampled immediately. Affected animals should be kept (isolated) as far away as possible from unaffected animals until the cause of the mortalities can be established.

F.1.1.2 *Surface Observations* (Level I)

Generally speaking, no surface observations can be linked to a single disease problem, however, quick detection of any of the following clinical signs, plus follow-up action (*e.g.*, removal or isolation from healthy fish, submission of samples for laboratory examination), can significantly reduce potential losses.

F.1.1.2.1 Skin and Fins (Level I)

Damage to the skin and fins can be the consequence of an infectious disease (*e.g.,* carp erythrodermatitis). However, pre-existing lesions due to mechanical damage from contact with rough surfaces, such as concrete raceways, or predator attack (*e.g.*, birds, seals, *etc.*, or chemical trauma) can also provide an opportunity for primary pathogens or secondary pathogens (*e.g.,* motile aeromonads) to invade and establish. This further compromises the health of the fish.

Common skin changes associated with disease, which should encourage further action include red spots (Fig. F.1.1.2.1a), which may be pin-point size (petechiae) or larger patches. These tend to occur around the fins, operculum, vent and caudal area of the tail, but may sometimes be distributed over the entire surface. Indications of deeper haemorrhaging or osmotic imbalance problem saredarkened colouration. Haemorrhagic lesions may precede skin erosion, which seriously affect osmoregulation and defense against secondary infections. Erosion is commonly found on the dorsal surfaces (head and back) and may be caused by disease, sunburn or mechanical damage. In some species, surface irritation may be indicated by a build up of mucous or scale loss.

Surface parasites, such as copepods, ciliates or flatworms, should also be noted. As with the gills, these may not be a problem under most circumstances, however, if they proliferate to noticeably higher than normal numbers (Fig. F.1.1.2.1b), this may lead to secondary infections or indicate an underlying disease (or other stress) problem. The parasites may be attached superficially or be larval stages encysted in the fins, or skin. Such encysted larvae (*e.g.*, flatworm digenean metacercariae) may be detected as white or black spots (Fig. F.1.1.2.1c)

in the skin (or deeper muscle tissue).

Abnormal growths are associated with tumourous diseases, which can be caused by disease, such as *Oncorhynchus masou* virus (see F.4 - *Oncorhynchus masou* Virus Disease) and Lymphocystis (see F.9 - Lymphocystis), or other environmental problems.

The eyes should also be observed closely for disease indications. Shape, colour, cloudiness, gas bubbles and small haemorrhagic lesions (red spots) can all indicate emerging or actual disease problems. For example, eye enlargement and distension, known as "Popeye", is associated with several diseases (Fig. F.1.1.2.1d).

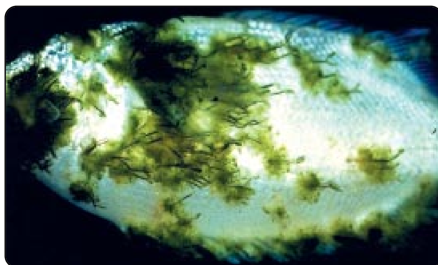
F.1.1.2.2 Gills (Level I)

The most readily observable change to soft tissues is paleness and erosion of the gills (Fig.F.1.1.2.2a). This is often associated with disease and should be of major concern. Red spots may also be indicative of haemorrhagic problems, which reduce the critical functioning ability of the gills. Fouling, mucous build-up or parasites (ciliate protistans, monogeneans, copepods, fungi, *etc.*) may also reduce functional surface area and may be indicative of other health problems (Fig.F.1.1.2.2b).These may affect the fish directly or render it more susceptible to secondary infections.

(MG Bondad-Reantaso)

Fig.F.1.1.2.1a. Red spot disease of grass carp.

(JR Arthur)



(K Ogawa)

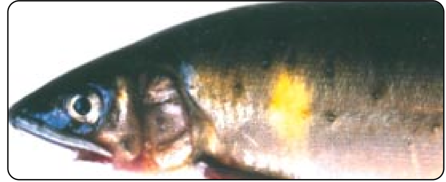


Fig.F.1.1.2.1c. Ayu, *Plecoglossus altivelis*, infected with *Posthodiplostomum cuticola* (?) metacercariae appearing as black spots on skin.

(R Chong)

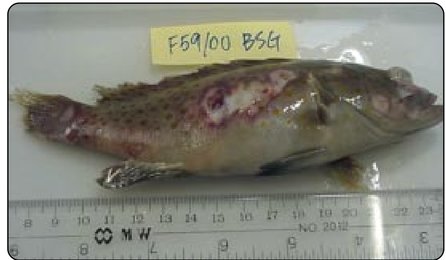


Fig. F.1.1.2.1d. Typical ulcerative, popeye, fin and tail rot caused by *Vibrio spp.*

(SE McGladdery)

Fig.F.1.1.2.2a. Example of gill erosion on Atlantic salmon, *Salmo salar*, due to intense infestation by the copepod parasite *Salmincola salmoneus.*

(MG Bondad-Reantaso)

Fig.F.1.1.2.2b. Fish gills infected with monogenean parasites.

Fig.F.1.1.2.1b. Surface parasites, *Lerneae cyprinacea* infection of giant gouramy.

F.1.1.2.3 Body (Level I)

Any deviation from normal body shape in a fish is a sign of a health problem. Common changes include "pinhead" which usually affects young fish indicating developmental problems; lateral or dorso-vental bends in the spine (*i.e.*, lordosis and scoliosis) can reveal nutritional or environmental water quality problems. Another common, and easily detected, change in body shape is "dropsy". Dropsy is a distention of the abdomen, giving the fish a "pot belly" appearance. This is a strong indicator of disease problems which may include swelling of internal organs (liver, spleen or kidney), build up of body fluids (clear = oedema; bloody fluids = ascites), parasite problems, or other unknown cause. Dropsy is a common element in many of the serious diseases listed in the *Asia Diagnostic Guide* since it is commonly associated with systemic disruption of osmoregulation due to blood-cell or kidney damage.

F.1.1.3 *Internal Observations* (Level I)

As a follow up to behavioural changes, samples of sick fish should be examined and cut open along the ventral surface (throat to anus). This will allow gross observation of the internal organs and body cavity. A healthy-appearing fish should also be opened up the same way, if the person has little experience with the normal internal workings of the fish they are examining. Organ arrangement and appearance can vary between species.

Normal tissues should have no evidence of free fluid in the body cavity, firm musculature, cream-white fat deposits (where present) around the pyloric caecae, intestine and stomach, a deep red kidney lying flat along the top of the body cavity (between the spinal cord and swim-bladder), a red liver, a deep red spleen and pancreas. The stomach and intestine may contain food. Gonadal development will vary depending on season. The heart (behind the gill chamber and walled off from the body cavity) and associated bulbous arteriosus should be distinct and shiny.

F.1.1.3.1 Body Cavity and Muscle (Level I/II)

Clues to disease in a body cavity most commonly consist of haemorrhaging and a build up of bloody fluids. Blood spots in the muscle of the body cavity wall, may also be present. Body cavity walls which disintegrate during dissection may indicate a fish that has been dead for a while and which is, therefore, of little use for accurate diagnosis, due to rapid invasion of secondary saprobionts (*i.e.,* microbes that live on dead and decaying tissues).

Necrotic musculature may also indicate a muscle infection, *e.g.*, by myxosporean parasites. This can be rapidly investigated by squashing a piece of the affected muscle between two glass slides or between a Petri dish lid and base, and examining it under a compound or dissection microscope. If spore-like inclusions are present, a parasite problem can be reasonably suspected. Some microsporidian and myxosporean parasites can form cysts in the muscle (Fig.F.1.1.3.1a), peritoneal tissues (the membranous network which hold the organs in place in the body cavity), and organs that easily visible to the naked eye as clumps or masses of white spheres. These too, require parasitology identification. Worms may also be present, coiled up in and around the organs and peritoneal tissues. None of these parasites (though unsightly) are usually a disease-problem, except where present in massive numbers which compress or displace the organs (Fig.F.1.1.3.1b).

F.1.1.3.2 Organs (Levels I-III)

Any white-grey patches present in the liver, kidney, spleen or pancreas, suggest a disease problem, since these normally represent patches of necrosis or other tissue damage. In organs such as kidney and spleen, this can indicate disruption of blood cell production. Kidney lesions can also directly affect osmoregulation and liver lesions can affect toxin and microbial defense mechanisms. Swelling of any of these organs to above normal size is equally indicative of a disease problem which should be identified, as soon as possible.

Swollen intestines (Fig.F.1.1.3.2a and Fig.F.1.1.3.2b) should be checked to see if this is due to food or a build up of mucous. The latter is indicative of feed and waste disposal disruption, as well as intestinal irritation, and is commonly found in association with several serious diseases. This may also occur due to opportunistic invasion of bowels that have been irritated by rapid changes in feed, *e.g.*, by the flagellate protistan *Hexamita salmonis*. Mucous filled intestines can be spotted externally via the presence of trailing, flocculent or mucous faeces (casts).

(H Yokoyama)

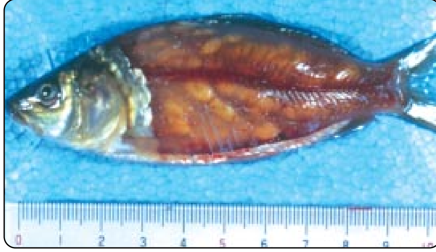


Fig.F.1.1.3.1a. *Myxobolus artus* infection in the skeletal muscle of 0+ carp.

(K Ogawa)

Fig.F.1.3.1b. *Ligula* sp. (Cestoda) larvae infection in the body cavity of Japanese yellow goby, *Acanthogobius flavimanus.*

(H Yokoyama)

Fig.F.1.3.2a. Distended abdomen of goldfish.

(MG Bondad-Reantaso)

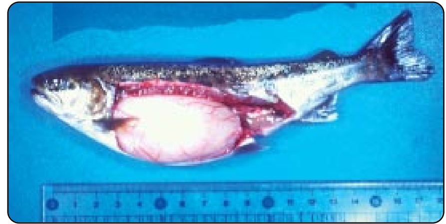


Fig.F.1.3.2b. Japanese Yamame salmon (*Onchorynchus masou*) fingerlings showing swollen belly due to yeast infection.

F.1.2 Environmental Parameters (Level I)

Water quality and fluctuating environmental conditions, although not of contagious concern, can have a significant effect on finfish health, both directly (within the ranges of physiological tolerances) and indirectly (enhancing susceptibility to infections). This is especially important for species grown in conditions that bear little resemblance to the wild situation. Water temperature, salinity, turbidity, fouling and plankton blooms are all important factors. High stocking rates, common in intensive aquaculture, predispose individuals to stress as well as minor changes in environmental conditions that can precipitate disease. Accumulation of waste feed indicates either overfeeding or a decrease in feeding activity. In either situation, the breakdown products can have a direct toxic effect or act as a medium for microbial proliferation and secondary infections. Likewise, other pollutants can also have a significant effect on fish health.

F.1.3 General Procedures

F.1.3.1 *Pre-Collection Preparation* (Level I)

Wherever possible, the number of specimens required for laboratory examination should be confirmed *before* the samples are collected. Larger numbers are generally required for screening purposes than for diagnosis of mortalities, or other abnormalities. The diagnostic laboratory which will be receiving the sample should also be consulted to ascertain the best method of transportation (*e.g.*, on ice, preserved in fixative, whole or tissue samples). The laboratory will also indicate if both clinically affected, as well as apparently healthy individuals, are required for comparative purposes.

Inform the laboratory of exactly what is going to be sent (*i.e.*, numbers, size-classes or tissues and intended date of collection and delivery) so the laboratory can be prepared *prior* to sample arrival. Such preparation can speed up processing of a sample (fixative preparation, labeling of slides, jars, cassettes, test-tubes, Petri-plates, data-sheets, *etc*.) by as much as a day.

F.1.3.2 *Background Information* (Level I)

All samples submitted for diagnosis should include as much supporting information as possible including:

- reason(s) for submitting the sample (*i.e.* health screening, certification)
- gross observations,feed records,and envronmental parameters
- history and origin of the fish population date of transfer and source location(s) if the stock does not originate from on-site.

These information will help clarify whether handling stress, change of environment or infectious agents are causes for concern. It will also help speed up diagnosis, risk assessment, and husbandry management and treatment recommendations.

F.1.3.3 *Sample Collection for Health Surveillance*

The most important factors associated with collection of specimens for surveillance are:

- sample numbers that are high enough (see Table F.1.3.3 below)
- susceptible species are sampled
- sampling includes age-groups and seasons that are most likely to manifest detectable infections.
  Such information is given under the specific disease sections.

	Prevalence (%)						
Population Size	0.5	1.0	**2.0**	3.0	4.0	**5.0**	10.0
50	46	46	46	37	37	29	20
100	93	93	76	61	50	43	23
250	192	156	110	75	62	49	25
500	314	223	127	88	67	54	26
1000	448	256	136	92	69	55	27
2500	512	279	142	95	71	56	27
5000	562	288	145	96	71	57	27
10000	579	292	146	96	72	29	27
100000	594	296	147	97	72	57	27
1000000	596	297	147	97	72	57	27
>1000000	600	300	**150**	100	75	**60**	30

Table F.1.3.3[1] . Sample sizes needed to detect at least one infected host in a population of a given size, at a given prevalence of infection. Assumptions of 2% and 5% prevalences are most commonly used for surveillance of presumed exotic pathogens, with a 95% confidence limit.

F.1.3.4 *Sample Collection for Disease Diagnosis* (Level I)

All samples submitted for disease diagnosis should include as much supporting information as possible including:

- reason(s) for submitting the sample (mortalities, abnormal growth, *etc.*)
- handling activities (net/cage de-fouling, size sorting/grading, site changes, predators, new species/stock introduction, *etc.*)

[1] Ossiander, F.J. and G. Wedermeyer. 1973. Journal Fisheries Research Board of Canada 30:1383-1384.

- environmental changes (*rapid* water quality changes, such as turbidity fluxes, saltwater incursion into freshwater ponds, unusual weather events, *etc.*).

These information will help clarify whether handling stress, change of environment or infectious agents may be a factor in the observed abnormalities/mortalities. Such information is necessary for both rapid and accurate diagnosis, since it helps focus the investigative procedures required.

F.1.3.5 *Live Specimen Collection for Shipping* (Level I)

Collection should take place as close to shipping time as possible, to reduce mortalities during transportation. This is especially important for moribund or diseased fish.

The laboratory should be informed of the estimated time of arrival of the sample, in order to ensure that the laboratory has the materials required for processing prepared before the fish arrive. This shortens the time between removal of the fish from water and preparation of the specimens for examination (see F.1.3.1).

The fish should be packed in double plastic bags, filled with water to one third of their capacity with the remaining 2/3 volume inflated with air/oxygen. The bags should be tightly sealed (rubber bands or tape) and packed inside a styrofoam box or cardboard box lined with styrofoam. A plastic bag measuring 60 x 180 cm is suitable for a *maximum* of *four* 200-300 g fish. The volume of water to fish volume/biomass is particularly important for live fish being shipped for ectoparasite examination, so advance checking with the diagnostic laboratory is recommended. The box should be sealed securely to prevent spillage and may be double packed inside a cardboard carton. The laboratory should be consulted about the packaging required.

Containers should be clearly labeled as follows:

"LIVE SPECIMENS, STORE AT ___ to ___°C, <u>DO NOT FREEZE"</u>
(Insert temperature tolerance range of fish being shipped)

If being shipped by air also indicate

"HOLD AT AIRPORT AND CALL FOR PICK-UP"
(Clearly indicate the name and telephone number of the person responsible for picking up the package, or receiving it at the laboratory).

Where possible, ship ***early in the week*** to avoid delivery during the weekend which may lead to improper storage and loss of samples.

Inform the contact person(s) as soon as the shipment has been sent and provide the name of the carrier, flight number, waybill number and estimated time of arrival, as appropriate.

F.1.3.6 *Dead or Tissue Specimen Collection for Shipping* (Level I)

In some cases, samples may be unable to be delivered live to a diagnostic laboratory due to distance or slow transport connections. In such cases, diagnostic requirements should be discussed with laboratory personnel prior to sample collection. Shipping of non-preserved tissues or dead specimens may require precautions to prevent contamination or decay. In addition, precautions should be taken to protect ectoparasites, if these are of probable significance.

For bacteriology, mycology or virology:

- Small fish may be bagged, sealed and transported whole on ice/frozen gel-packs.
- For larger fish, the viscera can be aseptically removed, placed in sterile containers and shipped on ice/frozen gel-packs.
- For bacteriology or mycology examinations – ship fish individually bagged and sealed, on ice/frozen gel-packs.
- For virology examination - bag fish with five volumes of Hanks' basal salt solution containing either gentamycin (1,000 mg/ml) or penicillin (800 IU/ml) + dihydrostreptamycin (800 mg/ml). Anti-fungal agents such as Mycostatin or Fungizone may also be incorporated at a level of 400 IU/ml.

Note: Intact or live specimens are ideally best since dissected tissues rapidly start autolysis even under ice, making them useless for sterile technique and bacteriology, particularly for tropical climates. Fish destined for bacteriological examination can be kept on ice for a ***limited*** period. The icing should be done to ensure that the organs/tissues destined for examination using sterile technique are kept at temperatures below ambient water (down to 4°C is a standard low) but not freezing. Individual bagging is also recommended in order to prevent contamination by

[2] Further details are available in "Recommendations for euthanasia of experimental animals" Laboratory Animals 31:1-32 (1997).

one individual within a sample.

F.1.3.7 *Preservation (Fixation) of Tissue Samples* (Level I)

Fish should be killed prior to fixation. With small fish, this can be done by decapitation, however, this causes mechanical damage to the tissues and is unsuitable for larger fish. Alternatively, euthanasia with an overdose of anaesthetic is a better (unless examination is for ectoparasites, which may be lost) option. Benzocaine or Etomidate, administered at triple the recommended dose is usually effective for anaesthesizing fish. Injection of anaesthetic should be avoided, wherever possible, due to handling induced tissue trauma[2]. Putting fish in iced water is also recommended prior to killing of fish.

Very small fish, such as fry or alevins, should be immersed directly in a minimum of 10:1 (fixative:tissue) volume ratio.

For large fish (>6 cm), the full length of the body cavity should be slit open (normally along the mid-ventral line) and the viscera and swim bladder gently displaced to permit incision of each major organ, at least once, to allow maximum penetration of the fixative. Ideally, the organ, or any lesions under investigation, should be removed, cut into blocks (<1.0 cm^3) and placed in a volume of fixative at least 10 times the volume of the tissue. Length of time for fixation is critical.
For skin sample preparation, it will be best to cut out several large pieces with a scalpel avoiding pressing or distortion of the sample. Briefly soak the skin in fixative, then take each piece of skin and cut into smaller sections about 1.0 cm wide and return the pieces quickly to fixative for 24 hrs. For samples from lesions, it is advisable to cut out a sample which includes healthy tissue surrounding the lesion to allow for comparison between healthy and affected tissues, with a width of no more than 1.0 cm and immediately placed in the fixative for 24 hrs.

Most tissues require a minimum of 24-48 hr fixation time if optimal preparations are to be made. It should be noted that long-term storage in all fixatives, except 70% ethanol, renders tissues useless for *in situ* hybridization. Check with diagnostic laboratory if long term storage is required on-site, prior to delivery to the laboratory.

The most suitable fixative for preservation of finfish samples for histopathology is **Phosphate Buffered Formalin.**

Phosphate Buffered Formalin

37-40% formaldehyde	100.0	ml
Tap water	900.0	ml
$NaH_2PO4.H_20$	4.0	g
Na_2HPO_4	6.5	g

Note: Formaldehyde is a gas soluble in water and is supplied in a concentrated form of 40% by weight. In concentrated solution, formaldehyde often becomes turbid during storage due to the production of formaldehyde, thus warming the solution or adding a small amount of NaOH will aid depolymerization of the paraformaldehyde. Formaldehyde is not suitable for fixation in its concentrated form. All formaldehyde regardless of purity, will be acid when purchased (usually within the pH range of 3-5). Care should be taken to check the final pH of any formalin-based fixative.

F.1.3.8 *Shipping Preserved Samples* (Level I)

Samples should be transported in sealed, unbreakable, containers. It is usual to double pack samples (*i.e.* an unbreakable container within a second unbreakable or well-padded container). Many postal services and transport companies (especially air couriers) have strict regulations regarding shipping chemicals, including preserved samples. If the tissues have been adequately fixed (as described in F.1.3.7), most fixative or storage solution can be drained from the sample for shipping purposes. As long as sufficient solution is left to keep the tissues from drying out, this will minimise the quantity of chemical solution being shipped. The carrier should be consulted before samples are collected to ensure they are processed and packed according to shipping rules.

- Containers should be clearly labeled with the information described for live specimens (F.1.3.5).
- The name and telephone number of the person responsible for picking up the package, or receiving it at the laboratory, should be clearly indicated.
- Where possible, ship early in the week to avoid delivery at the weekend, which may lead to improper storage and loss of samples.
- Inform the contact person as soon as the shipment has been sent and provide the name of the carrier, flight number, waybill number and

estimated time of arrival, as appropriate.

F.1.4 **Record-Keeping** (Level I)

It is critical to establish, and record, normal behaviour and appearance to compare with observations made during disease events. Record-keeping is, therefore, an essential component of *effective* disease management. For fish, many of the factors that should be recorded on a regular basis are outlined in sections F.1.4.1, F.1.4.2 and F.1.4.3.

F.1.4.1 *Gross Observations* (Level I)

These can be included in routine records of fish growth that, ideally would be monitored on a regular basis, either by sub-sampling from tanks or ponds, or by estimates made from surface observations.

For hatcheries, critical information that should be recorded include:

- feeding activity
- growth
- mortalities

These observations should be recorded daily, for all stages, including date, time, tank #, broodstock (where there are more than one) and food source. Dates and times of tank and water changes, pipe flushing/back-flushing and/or disinfection, should also be recorded. Ideally, these records should be checked (signed off) regularly by the person responsible for maintaining the facility.

For pond or net/cage sites, observations which need to be recorded include:

- growth
- fouling
- mortalities

These should be recorded with date, site location and any relevant activities (*e.g.*, sample collection for laboratory examination). As elsewhere, these records should be checked regularly by the person responsible for the facility.

F.1.4.2 *Environmental Observations* (Level I)

Environmental observations are most applicable to open water, ponds, cage and net culture systems. Information that should be recorded include:

- weather
- water temperature
- oxygen
- salinity
- turbidity (qualitative evaluation or Secchi disc)
- algal blooms
- human activity (handling, neighbouring land use/water activities)
- pH

The frequency of these observations will vary with site and fish species. Where salinity or turbidity rarely vary, records may only be required during rainy seasons or exceptional weather conditions. Temperate climates may require more frequent water temperature monitoring than tropic climates. Human activity(ies) should also be recorded on an "as it happens" basis, since there may be time-lag effects. In all cases, date and time should be recorded, as parameters such as temperature and pH can vary markedly during the day, particularly in open ponds and inter-tidal sites.

It may not always be possible to monitor oxygen levels in the pond. However, the farmer should be aware that in open non-aerated ponds, oxygen levels are lowest in the early morning when plants (including algae) have used oxygen overnight. Photosynthesis and associated oxygen production will only commence after sunrise.

F.1.4.3 *Stocking Records* (Level I)

All movements of fish into and out of a hatchery or site should be recorded, including:

- the source of the broodstock/eggs/larvae/juveniles and their health certification
- the volume or number of fish
- condition on arrival
- date and time of delivery and name of person responsible for receiving the fish
- date, time and destination of stock shipped-out from a hatchery or site.

Such records are also applicable (but less critical) to movements between tanks, ponds, cages within a site. Where possible, animals from different sources should not be mixed. If mixing is unavoidable, keep strict records of which sources are mixed and dates of new introductions into the holding site or system.

F.1.5 **References**

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F.2 EPIZOOTIC HAEMATOPOIETIC NECROSIS (EHN)

F.2.1 **Background Information**

F.2.1.1 *Causative Agent*

Epizootic Haematopoietic Necrosis (EHN) is caused by a double-stranded DNA, non-enveloped Iridovirus known as Epizootic Haematopoeitic Necrosis Virus (EHNV). This virus shares at least one antigen with iridoviruses infecting sheatfish (*Silurus glanis*) and the catfish (*Ictalurus melas*) in Europe and with amphibian iridoviruses from North America (frog virus 3) and Australia (Bohle iridovirus). Recently, the OIE included the two agents, European catfish virus and European sheatfish virus, as causative agents of EHN (OIE 2000a; http://www.oie.int). Current classification in the genus *Ranavirus* is under review (see http://www.ncbi.nlm.nih.gov/ICTV). More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

F.2.1.2 *Host Range*

EHNV infects redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*). Other fish species found to be susceptible to EHNV after bath exposure are Macquarie perch (*Macquaria australasica*), mosquito fish (*Gambussia affinis*), silver perch (*Bidyanus bidyanus*) and mountain galaxias (*Galaxias olidus*).

F.2.1.3 *Geographic Distribution*

Historically, the geographic range of EHNV infections has been restricted to mainland Australia. However, a recent OIE decision to include sheatfish and catfish iridoviruses as causes of EHN, increased the geographic distribution to include Europe. A related virus recently isolated from pike-perch in Finland, was found to be immunologically cross-reactive but non-pathogenic to rainbow trout.

F.2.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

Australia reported the occurrence of EHN in Victoria (last year 1996), New South Wales (last year 1996) and South Australia (1992). It was also known to have occurred in New South Wales during first quarter of 2000, with annual occurrence in the Australian Capital Territory (without laboratory confirmation) (OIE 1999, 2000b).

India reported EHN during last quarter of 1999 affecting murrels and catfishes (OIE 1999).

F.2.2 **Clinical Aspects**

There are no specific clinical signs associated with EHN. Mortalities are characterised by necrosis of liver (with or without white spots), spleen, haematopoietic tissue of the kidney and other tissues. Disruption of blood function leads to osmotic imbalance, haemorrhagic lesions, build up of body fluids in the body cavity. The body cavity fluids (ascites) plus enlarged spleen and kidney may cause abdominal distension (dropsy).

Clinical disease appears to be associated with poor water quality, as well as water temperature. In rainbow trout, disease occurs at temperatures from 11 to 17°C (in nature) and 8 - 21°C (experimental conditions). No disease is found in redfin perch at temperatures below 12°C under natural conditions. Both juvenile and adult redfin perch can be affected, but juveniles appear more susceptible (Fig.F.2.2a). EHNV has been detected in rainbow trout ranging from fry to market size, although mortality occurs most frequently in 0+ - 125 mm fork-length fish.

F.2.3 **Screening Methods**

More detailed information on methods for screening EHN can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or selected references.

As with other disease agents, screening for the presence of an infectious agent in a sub-clinical population requires larger sample numbers than for a disease diagnosis. Numbers will vary according to the confidence level required (see F.1.3.3).

F.2.3.1 *Presumptive*

F.2.3.1.1 Gross Observations (Level 1) and Histopathology (Level II)

It is not possible to detect infections in sub-clinical fish, using gross observations (Level I) or histopathology (Level II).

F.2.3.1.2 Virology (Level III)

EHNV can be isolated on Bluegill Fin 2 (BF-2) or Fathead Minnow (FHM) cell lines. This requires surveillance of large numbers (see Table F.1.3.3)

of sub-clinical fish to detect low percentage carriers.

F.2.3.2 *Confirmatory*

F.2.3.2.1 Immunoassays (Level III)

Suspect cytopathic effects (CPE) in BF-2 or FHM cell-lines require confirmation of EHNV as the cause through immunoassay (indirect fluorescent antibody test (IFAT) or enzyme linked immunosorbent assay (ELISA) or Polymerase Chain Reaction (PCR) (Level III).

F.2.4 **Diagnostic Methods**

More detailed information on methods for diagnosis of EHN can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or selected references.

EHNV is a highly resistant virus that can withstand freezing for prolonged periods, thus, fish may be stored and or/transported frozen without affecting the diagnosis.

F.2.4.1 *Presumptive*

F.2.4.1.1 Gross Observation (Level I)

As described under F.2.2, mass mortalities of small redfin perch under cool water conditions (< 11 °C), which include cessation of feeding, abdominal distension, focal gill and fin haemorrhage, as well as overall skin darkening, should be considered suspect for EHNV infection. Similar observations in rainbow trout fingerlings (11-17 °C) may also be considered suspect, but the conditions are not specific to EHN in either host.

Necropsy may reveal liver and spleen enlargement or focal pale spots on the liver, but these, again, are non-specific.

F.2.4.1.2 Histopathology (Level II)

Histopathology in haematopoietic kidney, liver, spleen and heart tissues are similar in both infected redfin perch and rainbow trout, although perch livers tend to have larger focal or locally extensive areas of necrosis. Gills of infected perch show focal blood clots, haemorrhage and fibrinous exudate. Focal necrosis occurs in the pancreas and intestinal wall. In the former tissue site necrosis can become extensive.

F.2.4.1.3 Virology (Level III)

Whole alevin or juvenile perch (<4 cm in length), viscera including kidney (4-6 cm body length) or kidney, spleen and liver from larger fish, are required for tissue culture. Presumptive diagnosis starts with viral isolation on BF-2 or FHM cell-lines. Cytopathic effect (CPE) is then cross-checked for EHNV using indirect fluorescence microscopy or ELISA (F.2.4.2).

F.2.4.1.4 Transmission Electron Microscopy (TEM) (Level III)

Icosahedral morphology, 145-162 *nm*, dsDNA non-enveloped viral particles are present in the cytoplasm of infected spleen, liver, kidney and blood cells.

F.2.4.2 *Confirmatory*

F.2.4.2.1 Immunoassay (Level III)

IFAT and ELISA are required to confirm EHNV in CPE from cell-line culture described under F.2.4.1.3. EHNV does not induce neutralising antibodies (Ab) in mammals or fish.

F.2.4.2.2 Polymerase Chain Reaction (PCR) (Level III)

PCR procedures and primers have been produced that can detect iridoviruses in isolates from redfin perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiss*), sheatfish (*Silurus glanis*), catfish (*Ictalurus melas*), guppy (*Poecilia reticulata*), doctor fish (*Labroides dimidatus*), and a range of amphibian ranaviruses (unpublished data).

F.2.5 **Modes of Transmission**

Transmission of EHNV in rainbow trout is not fully understood. Infections may recur annually and this may be linked to redfin perch reservoirs of infection in the water catchment area. However, the disease is also known to occur at low prevalences in some infected trout populations, so mortality may not exceed “normal” background rates. This means infected fish may be overlooked among apparently healthy fish.

Another route of EHNV spread is by birds, either by regurgitation of infected fish, or mechanical transfer on feathers, feet or beaks. Anglers have also been implicated in EHNV transfer, either via dead fish or by contaminated fishing gear.

F.2.6 Control Measures

Prevention of movement of infected fish between watersheds, and minimising contact between trout farms and surrounding perch populations is recommended. In addition, reducing bird activity at farm sites may be effective in reducing the chances of exposure and spread. Precautionary advice and information for recreational fishermen using infected and uninfected areas may also reduce inadvertent spread of EHN.

F.2.7 Selected References

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(AAHL)

Fig.F.2.2a. Mass mortality of single species of redfin perch. Note the small size of fish affected and swollen stomach of the individual to the centre of the photograph. Note the characteristic haemorrhagic gills in the fish on the left in the inset.

(EAFP)

Fig.F.3.2a. IHN infected fry showing yolk sac haemorrhages.

(EAFP)

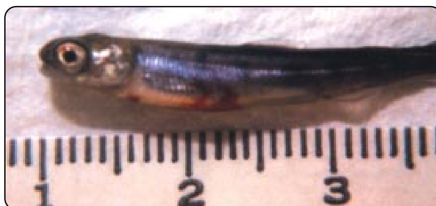

Fig.F.3.2b. Clinical signs of IHN infected fish include darkening of skin, haemorrhages on the abdomen and in the eye around the pupil.

F.3 INFECTIOUS HAEMATOPOIETIC NECROSIS (IHN)

F.3.1 **Background Information**

F.3.1.1 *Causative Agent*

Infectious Haematopoietic Necrosis (IHN) is caused by an enveloped single stranded RNA (ssRNA) Rhabdovirus, known as Infectious Haematopoietic Necrosis Virus (IHNV). It is currently unassigned to genus, but the International Committee on Taxonomy of Viruses (ICTV) is currently reviewing a new genus – Novirhabdovirus – which is proposed to include VHSV and IHNV (see http://www.ncbi.nlm.nih.gov/ICTV). More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

F.3.1.2 *Host Range*

IHNV infects rainbow or steelhead trout (*Oncorhynchus mykiss*), sockeye salmon (*O. nerka*), chinook (*O. tshawytscha*), chum (*O.keta*), yamame (*O. masou*), amago (*O. rhodurus*), coho (*O. kisutch*), and Atlantic salmon (*Salmo salar*). Pike fry (*Esox lucius*), seabream and turbot can also be infected under experimental conditions.

F.3.1.3 *Geographic Distribution*

Historically, the geographic range of IHN was limited to the Pacific Rim of North America but, more recently, the disease has spread to continental Europe and Asia.

F.3.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999- 2000)*

India reported occurrence of IHN during last quarter of 1999 affecting murrels and catfishes; Korea RO reported IHN among rainbow trout during 3rd and 4th (September) quarters of 2000 while Japan reported occurrence of IHN every month during 1999 and 2000 (OIE 1999, 2000b).

F.3.2 **Clinical Aspects**

Among individuals of each fish species, there is a high degree of variation in susceptibility to IHNV. Yolk-sac fry (Fig.F.3.2a) are particularly susceptible and can suffer 90-100% mortality. In rainbow trout, such mortalities are correlated with water temperatures <14°C. Survivors of IHNV demonstrate strong acquired immunity.

Susceptible fish show dark discolouration of the body (especially the dorsal surface and tail fin regions) (Fig.F.3.2b). The abdomen may be distended, with haemorrhaging at the base of the fins, on the operculum and around the eyes (which may show swelling – "pop-eye"). Weakened swimming capability may also be evident. Some fish may show a white discharge from the anus.

The IHNV multiplies in endothelial cells of blood capillaries, spleen and kidney cells, which results in osmotic imbalance, as well as systemic haemorrhagic lesions. These can be seen grossly as pale internal organs and/or pin-point bleeding in the musculature and fatty tissues. The kidney, spleen, brain and digestive tract are the sites where virus is most abundant during advanced infection.

F.3.3 **Screening Methods**

More detailed information on methods for screening IHN can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

F.3.3.1 *Presumptive*

F.3.3.1.1 Virology (Level III)

IHNV can be isolated from sub-clinical carriers on *Epithelioma papulosum cyprinae* (EPC) or BF-2 cell lines. The identity of the cause of any CPE on these cell lines, however, requires further confirmation (F.3.3.2).

F.3.3.2 *Confirmatory*

F.3.3.2.1 Immunoassay or Nucleic Acid Assay (Level III)

The cause of CPE produced on EPC or BF-2 cell lines by suspect IHNV carrier samples must be confirmed using immunological identification or PCR-based techniques (F.3.4.2.1).

F.3.4 **Diagnostic methods**

More detailed information on methods for diagnosis of IHN can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

F.3.4.1 *Presumptive*

F.3.4.1.1 Gross Observations (Level I)

Behavioural changes are not specific to IHN but may include lethargy, aggregation in still areas of the pond with periodic bursts of erratic swimming (see F.3.2) and loss of equilibrium.

Changes in appearance include dark discolouration of the body (especially the dorsal surface and tail fin regions), especially in yolk-sac fry stages (90-100% mortality). The abdomen can be distended due to accumulation of fluids in the body cavity (dropsy) and haemorrhaging may be visible at the base of the fins, on the operculum and around the eyes. The eyes may also show signs of water imbalance in the tissues by bulging ("pop-eye"). There may be vent protrusion and trailing white/mucoid casts.

F.3.4.1.2 Histopathology (Level II)

Tissue sections show varying degrees of necrosis of the kidney and spleen (haematopoietic) tissues, as well as in the brain and digestive tract.

F.3.4.1.3 Virology (Level III)

Whole alevins (body length ≤4 cm), viscera including kidney (fish 4 – 6 cm in length) or kidney, spleen and brain tissues from larger fish, are required for isolating the virus on EPC or BF-2 cell lines. Confirmation of IHNV being the cause of any resultant CPE requires immunoassay investigation, as described below.

F.3.4.2 *Confirmatory*

F.3.4.2.1 Immunoassays (IFAT or ELISA) (Level III)

Diagnosis of IHNV is achieved via immunoassay of isolates from cell culture using IFAT or ELISA, or immunological demonstration of IHNV antigen in infected fish tissues.

F.3.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

TEM of cells infected in cell-culture reveals enveloped, slightly pleomorphic, bullet shaped virions, 45-100 *nm* in diameter and 100-430 *nm* long. Distinct spikes are evenly dispersed over most of the surface of the envelope (although these may be less evident under some cell-culture conditions). The nucleocapsids are coiled and show cross-banding (4.5 – 5.0 *nm* apart) in negative stain and TEM. Viral replication takes place in the cytoplasm with particle maturation at the cell membrane or the Golgi cisternae.

F.3.5 **Modes of Transmission**

IHNV is usually spread by survivors of infections, which carry sub-clinical infections. When such fish mature, they may shed the virus during spawning. Clinically infected fish can also spread the disease by shedding IHNV with faeces, urine, spawning fluids and mucus secretions. Other sources of infection include contaminated equipment, eggs from infected fish, and blood sucking parasites (*e.g.*, leeches, *Argulus* spp.). Fish-eating birds are believed to be another mechanism of spread from one site to another.

The most prominent environmental factor affecting IHN is water temperature. Clinical disease occurs between 8°C and 15°C under natural conditions. Outbreaks rarely occur above 15°C.

F.3.6 **Control Measures**

Control methods currently rely on avoidance through thorough disinfection of fertilised eggs. Eggs, alevins and fry should be reared on virus-free water supplies in premises completely separated from possible IHNV-positive carriers. Broodstock from sources with a history of IHN outbreaks should also be avoided wherever possible. At present, vaccination is only at an experimental stage.

As with viral haemorrhagic septicaemia virus (VHSV, see F.8), good over-all fish health condition seems to decrease the susceptibility to overt IHN, while handling and other types of stress frequently cause sub-clinical infection to become overt.

F.3.7 **Selected References**

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F.4 *ONCORHYNCHUS MASOU* VIRUS (OMV)

F.4.1 **Background Information**

F.4.1.1 *Causative Agent*

Oncorhynchus masou virus disease (OMVD) is caused by *Oncorhynchus masou* virus (OMV) is believed to belong to the Family *Herpesviridae*, based on an icosahedral diameter of 120-200 *nm*, and enveloped, dsDNA properties. OMV is also known as Yamame tumour virus (YTV), Nerka virus Towada Lake, Akita and Amori prefecture (NeVTA), coho salmon tumour virus (CSTV), *Oncorhynchus kisutch* virus (OKV), coho salmon herpesvirus (CSHV), rainbow trout kidney virus (RKV), or rainbow trout herpesvirus (RHV). OMV differs from the herpesvirus of Salmonidae type 1, present in the western USA. Currently this salmonid herpesvirus has not been taxonomically assigned (see http://www.ncbi.nlm.nih.gov/ICTV). More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

F.4.1.2 *Host Range*

Kokanee (non-anadromous sockeye) salmon (*Oncorhynchus nerka*) is most susceptible, followed, in decreasing order of susceptibility, by masou salmon (*O. masou*), chum salmon (*O. keta*), coho salmon (*O. kisutch*) and rainbow trout (*O. mykiss*).

F.4.1.3 *Geographic Distribution*

OMVD is found in Japan and, probably (as yet undocumented) the coastal rivers of eastern Asia that harbour Pacific salmon.

F.4.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

Japan reported OMVD during all months of 1999 and 2000; and suspected by Korea RO for 1999, and during first two quarters of 2000 (OIE 1999, 2000b).

F.4.2 **Clinical Aspects**

OMV infects and multiplies in endothelial cells of blood capillaries, spleen and liver, causing systemic oedema and haemorrhaging. One-month-old alevins are the most susceptible development stage. Kidney, spleen, liver and tumours are the sites where OMV is most abundant during the course of overt infection. Four months after the appearance of clinical signs, some surviving fish may develop epitheliomas (grossly visible tumours) around the mouth (upper and lower jaw) and, to a lesser extent, on the caudal fin operculum and body surface. These may persist for up to 1 year. In 1 yr-old coho salmon, chronic infections manifest themselves as skin ulcers, white spots on the liver and papillomas on the mouth and body surface. In rainbow trout, however, there are few (if any) external symptoms, but intestinal haemorrhage and white spots on the liver are observed.

Survivors of OMVD develop neutralising antibodies which prevent re-infection, however, they can remain carriers of viable virus.

F.4.3 **Screening Methods**

More detailed information on screening methods for OMV can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

F.4.3.1 *Presumptive*

F.4.3.1.1 Gross Observations (Level I)

Persistent superficial tumours are rare, but indicative of a potential carrier of viable OMV. Species, such as rainbow trout show no such lesions. Sub-clinical carriage cannot normally be detected using histology.

F.4.3.1.2 Virology (Level III)

OMV can be isolated from reproductive fluids, kidney, brain and spleen tissue samples on Chinook salmon embryo-214 (CHSE-214) or rainbow trout gonad-2 (RTG-2) cell lines. Any resultant CPE requires further immunological and PCR analyses to confirm the identity of the virus responsible (see F.4.3.2.1).

F.4.3.2 *Confirmatory*

F.4.3.2.1 Immunoassays and Nucleic Acid Assays (Level III)

Cytopathic effect (CPE) from cell cultures, as well as analyses of reproductive fluids, kidney, brain and spleen tissue samples from suspect fish can be screened using specific neutralisation anti-body tests, indirect immunofluorescent anti-body tests (IFAT) with immunoperoxidase staining, ELISA or Southern Blot DNA probe assays.

F.4 *Oncorhynchus Masou* Virus (OMV)

F.4.4 **Diagnostic Methods**

More detailed information on diagnostic methods for OMV can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000b), at http://www.oie.int or selected references.

F.4.4.1 *Presumptive*

F.4.4.1.1 Gross Observation (Level I)

Behavioural changes include lethargy and aggregation around the water inflow by young salmonids of susceptible species. Pin-point haemorrhaging or ulcers may be visible on the skin, along with darkened colouration. Popeye may also be present. Internally, white spots may be present on the liver (Fig.F.4.4.1.1a). After approximately 4 months, surviving fish may show signs of skin growths around the mouth (Fig.F.4.4.1.1b) or, less commonly, on the operculum, body surface or caudal fin area.

(M Yoshimizu)

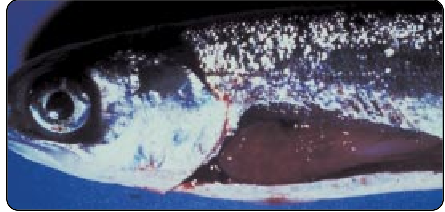

Fig.F.4.4.1.1a. OMV-infected chum salmon showing white spots on the liver.

(M Yoshimizu)

Fig.F.4.4.1.1b. OMV-induced tumour developing around the mouth of chum salmon fingerling.

F.4.4.1.2 Histopathology (Level II)

Tissue sections from suspect fish may show lesions with enlarged nuclei in the epithelial tissues of the jaw, inner operculum and kidney.

F.4.4.1.3 Virology (Level III)

Whole alevin (body length ≤ 4 cm), viscera including kidney (4 – 6 cm length) or, for larger fish, skin ulcerative lesions, neoplastic (tumourous tissues), kidney, spleen and brain are required for tissue culture using CHSE-214 or RTG-2 cell-lines. The cause of resultant CPE should be confirmed as viral using the procedures outlined in F.4.3.2.1.

F.4.4.1.4 Transmission Electron Microscopy (TEM) (Level III)

Detection of virions in the nuclei of affected tissues and tumours by TEM. The dsDNA virions are enveloped and icosahedral, measuring 120-200 *nm* in diameter (Fig.F.4.4.1.3).

(M Yoshimizu)

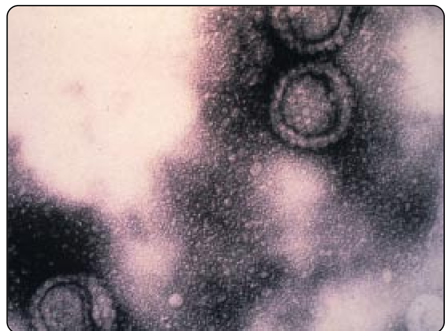

Fig.F.4.4.1.3. OMV particles isolated from masou salmon, size of nucleocapsid is 100 to 110 *nm*.

F.4.4.2 *Confirmatory*

F.4.4.2.1 Gross Observations (Level I)

Gross behaviour and clinical signs at the onset of OMVD are not disease specific. Thus, confirmatory diagnosis requires additional diagnostic examination or occurrence with a docu-

mented history of OMVD on-site or mortalities several months preceding the appearance of epithelial lesions and tumours.

F.4.4.2.2 Virology (Level III)

As described for F.4.3.1.2

F.4.4.2.3 Immunoassays and Nucleic Acid Assays (Level III)

As described for F.4.3.2.1.

F.4.5 Modes of Transmission

Virus is shed with faeces, urine, external and internal tumours, and, possibly, with skin mucus. Reservoirs of OMV are clinically infected fish as well as wild or cultured sub-clinical carriers. Maturation of survivors of early life-history infections may shed virus with their reproductive fluids ("egg associated", rather than true vertical transmission). Egg-associated transmission, although less frequent than other mechanisms of virus release, is the most likely source of infection in alevins.

F.4.6 Control Measures

Thorough disinfection of fertilised eggs, in addition to rearing of fry and alevins, in water free of contact with contaminated materials or fish, has proven effective in reducing outbreaks of OMVD.Water temperatures <14˚C appear to favour proliferation of OMV.

F.4.7 Selected References

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F.5 INFECTIOUS PANCREATIC NECROSIS (IPN)

F.5.1 **Background Information**

F.5.1.1 *Causative Agent*

Infectious pancreatic necrosis (IPN) is caused by a highly contagious virus, Infectious pancreatic necrosis virus (IPNV) belonging to the *Birnaviridae*. It is a bi-segmented dsRNA virus which occurs primarily in freshwater, but appears to be saltwater tolerant. More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

F.5.1.2 *Host Range*

IPN most commonly affects rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*) and several Pacific salmon species (*Oncorhynchus* spp.). Serologically related are reported from Japanese yellowtail flounder (*Seriola quinqueradiata*), turbot (*Scophthalmus maximus*), and halibut (*Hippoglossus hippoglossus*). Sub-clinical infections have also been detected in a wide range of estuarine and freshwater fish species in the families Anguillidae, Atherinidae, Bothidae, Carangidae, Cotostomidae, Cichlidae, Clupeidae, Cobitidae, Coregonidae, Cyprinidae, Esocidae, Moronidae, Paralichthydae, Percidae, Poecilidae, Sciaenidae, Soleidae and Thymallidae.

F.5.1.3 *Geographic Distribution*

The disease has a wide geographical distribution, occurring in most, if not all, major salmonid farming countries of North and South America, Europe and Asia.

F.5.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

IPN was reported by Japan and suspected in Korea RO for 1999; in 2000, reported by Japan for the whole year except for the month of February, and by Korea RO in April (OIE 1999, 2000b).

F.5.2 **Clinical Aspects**

The first sign of IPN in salmonid fry is the sudden onset of mortality. This shows a progressive increase in severity, especially following introduction of feed to post-yolk-sac fry. IPN also affects American salmon smolt shortly after transfer to sea-cages. Clinical signs include darkening of the lower third of the body and small swellings on the head (Fig.F.5.2.a) and a pronounced distended abdomen (Fig.F.5.2b and Fig.F.5.2c) and a corkscrewing/spiral swimming motion. Some fish may also show 'pop-eye' deformities. Cumulative mortalities may vary from less than 10% to more than 90% depending on the combination of several factors such as virus strain, host and environment. Survivors of the disease, at early or late juvenile stages, are believed to be carriers of viable IPNV for life. Mortality is higher when water temperatures are warm, but there is no distinct seasonal cycle.

The pancreas, oesophagus and stomach become ulcerated and haemorrhagic. The intestines empty or become filled with clear mucous (this may lead to white fecal casts).

F.5.3 **Screening Methods**

More detailed information on screening methods for IPN can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000b), at http://www.oie.int or selected references.

As with other disease agents, screening for the presence of an infectious agent in a sub-clinical population requires larger sample numbers than for a disease diagnosis. Numbers will vary according to the confidence level required (see F.1.3.3).

F.5.3.1 *Presumptive*

F.5.3.1.1 Gross Observations (Level I) and Histopathology (Level II)

Carriers of sub-clinical infections show no external or internal evidence of infection at the light microscope level.

F.5.3.1.2 Virology (Level III)

Screening procedures use viral isolation on Chinook Salmon Embryo (CHSE-214) or Bluegill Fin (BF-2) cell lines. The cause of any CPE, however, has to be verified using confirmatory techniques (F.5.3.2.2). Fish material suitable for virological examination include whole alevin (body length ≤ 4 cm), viscera including kidney (fish 4 – 6 cm in length) or, liver, kidney and spleen from larger fish.

F.5 Infectious Pancreatic Necrosis (IPN)

F.5.3.2 *Confirmatory*

F.5.3.2.1 Immunoassays and Molecular Probe Assays (Level III)

The viral cause of any CPE on CHSE-214 or BF-2 cell lines has to be confirmed by either an immunoassay (Neutralisation test or ELISA) or PCR techniques, including reverse-transcriptase PCR (RT-PCR) and *in situ* hybridization (ISH).

(EAFP)

Fig.F.5.2a. IPN infected fish showing dark colouration of the lower third of the body and small swellings on the head.

(J Yulin)

Fig.F.5.2b. Rainbow trout fry showing distended abdomen characteristic of IPN infection. Eyed-eggs of this species were imported from Japan into China in 1987.

(EAFP)

Fig.F.5.2c. Top: normal rainbow trout fry, below: diseased fry.

(J Yulin)

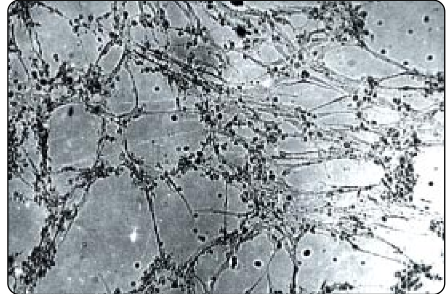

Fig.F.5.4.1.3. CPE of IHNV.

(J Yulin)

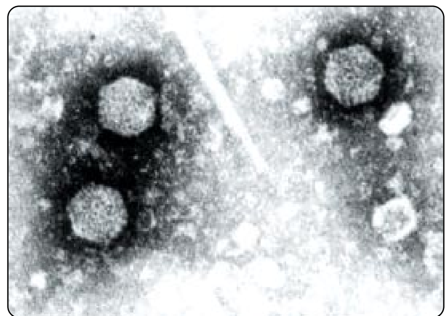

Fig.F.5.4.1.4. IPN Virus isolated from rainbow trout fimported from Japan in1987. Virus particles are 55 *nm* in diameter.

F.5.4 **Diagnostic Methods**

More detailed information on diagnostic methods for IPN can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000b), at http://www.oie.int or selected references.

F.5.4.1 *Presumptive*

F.5.4.1.1 Gross Observations (Level I)

Clinical signs in salmonid fry and parr include lying on the bottom of tanks/ponds, or showing cork-screw swimming behaviour. High mortalities may occur when fry are first fed or in smolt shortly after transfer to seawater. Chronic low mortalities may persist at other times. Dark discolouration (especially of the dorsal and tail surfaces) may be accompanied by swollen abdomens, pop-eye and/or pale faecal casts.

F.5.4.1.2 Histopathology (Level II)

Tissue pathology is characterised by necrotic lesions and ulcers in the pancreas, oesophagus and stomach. The intestines may be empty or filled with clear mucus (NB difference from parasite infection by *Hexamita inflata* (Hexamitiasis), where there is a yellowish mucus plug).

F.5.4.1.3 Virology (Level III)

As described for screening (F.5.3.1.2), fish material suitable for virological examination include whole alevin (body length ≤ 4 cm), viscera including kidney (fish 4 – 6 cm in length) or, liver, kidney and spleen for larger fish. The virus (Fig.F.5.4.1.3) can be isolated on CHSE-214 or BF-2 cell lines, but the cause of resultant CPE has to be verified using confirmatory techniques (F.5.3.2).

F.5.4.1.4 Transmission Electron Microscopy (TEM) (Level III)

The ultrastructural characteristics of IPNV are shared by most aquatic birnavididae, thus, immunoassay or nucleic acid assays are required for confirmation of identity. Birnaviruses are non-enveloped, icosahedral viruses, measuring approximately 60 *nm* in diameter (Fig.F.5.4.1.4). The nucleic acid component is bi-segmented, dsRNA, which can be distinguished using standard histochemistry.

F.5.4.2 *Confirmatory*

F.5.4.2.1 Virology and Immunoassay (Level III)

As described for screening (F.5.3.2.1), the viral cause of any CPE on CHSE-214 or BF-2 cell lines has to be confirmed by either an immunoassay (Neutralisation test or ELISA) or PCR techniques, including RT-PCR and ISH.

F.5.5 Modes of Transmission

The disease is transmitted both horizontally through the water route and vertically via the egg. Horizontal transmission is achieved by viral uptake across the gills and by ingestion. The virus shows strong survival in open water conditions and can survive a wide range of environmental parameters. This, in addition to its lack of host specificity, provides gives IPNV the ability to persist and spread very easily in the open-water environment.

F.5.6 Control Measures

Prevention methods include avoidance of fertilised eggs from IPNV carrier broodstock and use of a spring or borehole water supply (free of potential reservoir fish). Surface disinfection of eggs has not been entirely effective in preventing vertical transmission.

Control of losses during outbreaks involves reducing stocking densities and dropping water temperatures (in situations where temperature can be controlled).

Vaccines are now available for IPN and these should be considered for fish being grown in IPNV endemic areas.

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F.6 VIRAL ENCEPHALOPATHY AND RETINOPATHY (VER)

F.6.1 Background Information

F.6.1.1 *Causative Agents*

Viral Encephalopathy and Retinopathy (VER) is caused by icosahedral, non-enveloped nodaviruses, 25-30 *nm* in diameter. These agents are also known as Striped Jack Nervous Necrosis Virus (SJNNV), Viral Nervous Necrosis (VNN) and Fish Encephalitis Virus (FEV). All share serological similarities with the exception of those affecting turbot (F.6.1.2). More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

F.6.1.2 *Host Range*

The pathology of VER occurs in larval and, sometimes, juvenile barramundi (sea bass, *Lates calcarifer*), European sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*), Japanese parrotfish (*Oplegnathus fasciatus*), red-spotted grouper (*Epinepheles akaara*), and striped jack (*Pseudocaranx dentex*). Disease outbreaks with similar/identical clinical signs have been reported in tiger puffer (*Takifugu rubripes*), Japanese flounder (*Paralichthys olivaceus*), kelp grouper (*Epinephelus moara*), brown spotted grouper (*Epinephelus malabaricus*), rock porgy (*Oplegnathus punctatus*), as well as other cultured marine fish species.

F.6.1.3 *Geographic Distribution*

VER occurs in Asia, the Mediterranean and the Pacific.

F.6.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

Australia reported VER occurrence during 8 of 12 months in 1999, and 7 of 12 months in 2000. Japan reported VER in 6 of 12 months in 2000, and 3 of 12 months in 1999. Last major outbreak reported by Singapore was in 1997 and recently in April 1999 and November 2000 among seabass. Korea RO suspected VER occurrence for whole year of 1999 and half year of 2000 (OIE 1999, OIE 2000b).

F.6.2 Clinical Aspects

VER affects the nervous system. All affected species show abnormal swimming behaviour (cork-screwing, whirling, darting and belly-up motion) accompanied by variable swim bladder hyperinflation, cessation of feeding, changes in colouration, and mortality (Fig.F.6.2). Differences between species are most apparent with relation to age of onset and clinical severity. Earlier clinical onset is associated with greater mortality, thus onset at one day post-hatch in striped jack results in more severe losses than suffered by turbot, where onset is up to three weeks post-hatch. Mortalities range from 10-100%.

Two forms of VER have been induced with experimental challenges (Peducasse *et al*.1999):

i) acute – induced by intramuscular inoculation, and
ii) sub-acute – by intraperitoneal inoculation, bath, cohabitation and oral routes.

F.6.3 Screening Methods

More detailed information on screening methods for VER can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000b), at http://www.oie.int or selected references.

F.6.3.1 *Presumptive*

There are no obvious diagnostic lesions that can be detected in sub-clinical carriers.

F.6.3.2 *Confirmatory*

F.6.3.2.1 Virology (Level III)

The nodavirus from barramundi has been cultured on a striped snakehead (*Channa striatus*) cell line (SSN-1) (Frerichs *et al*. 1996). The applicability of this cell line to other nodaviruses in this group is unknown.

F.6.3.2.2 Nucleic Acid Assays (Level III)

A newly developed polymerase chain reaction (PCR) method has shown potential for screening potential carrier striped jack and other fish species (*O. fasciatus, E. akaara, T. rubripes, P. olivaceus, E. moara, O. punctatus* and *D. labrax*).

F.6.4 Diagnostic Methods

More detailed information on diagnostic methods for VER can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000b), at http://www.oie.int or selected references.

F.6 Viral Encephalopathy and Retinopathy (VER)

(J Yulin)

Fig.F.6.2. Fish mortalities caused by VER.

F.6.4.1 *Presumptive*

F.6.4.1.1 Gross Observations

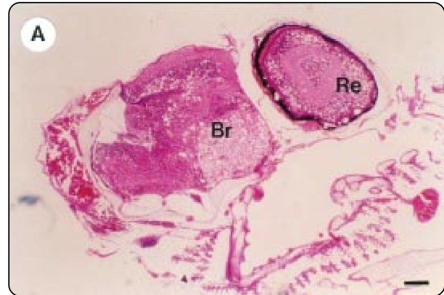
Abnormal swimming behaviour and swim-bladder inflation in post-hatch larvae and juvenile stages of the host. Species described above, along with associated mortalities are indicative of VER. Different species show different gross clinical signs (Table F.6.4.1.1). Non-feeding, wasting and colour changes in association with behavioural abnormalities, should also be considered suspect.

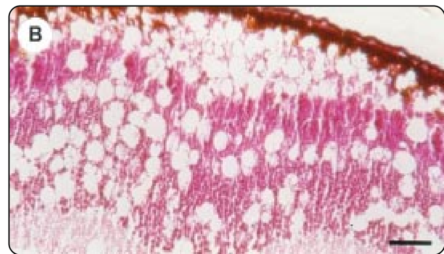
F.6.4.1.2 Histopathology (Level II)

Normal histological methods may reveal varying degrees of vacuolisation in the brain or retinal tissues (Fig.F.6.4.1.2a and Fig.F.6.4.1.2b). Small larvae can be embedded whole in paraffin blocks and serially sectioned to provide sections of brain and eyeballs. Larger fish (juvenile) usually require removal and fixation of eyes and brain.

All the diseases described/named under F.6.1.1 demonstrate vacuolisation of the brain, although some species (*e.g.*, shi drum, *Umbrina cirrosa*) may show fewer, obvious, vacuolar lesions. In addition, vacuolisation of the nuclear layers of the retina may not be present in Japanese parrotfish or turbot. Intracytoplasmic inclusions (≤ 5 *µm* diameter) have been described in sections of European sea bass and Australian barramundi, Japanese parrotfish and brown-spotted grouper nerve tissue. Neuronal necrosis has been described in most species. Vacuolisation of the gut is not caused by VER nodaviruses, but is typical.

(S Chi Chi)





Figs.F.6.4.1.2a, b. Vacuolation in brain (Br) and retina (Re) of GNNV-infected grouper in Chinese Taipei (bar = 100 mm).

F.6.4.2 *Confirmatory*

F.6.4.2.1 Virology (Level III)

As described under F.6.3.2.1.

F.6.4.2.2 Immunoassays (Level III)

Immunohistochemistry protocols for tissue sections fixed in Bouin's or 10% buffered formalin and direct fluorescent antibody test (DFAT) techniques use antibodies sufficiently broad in specificity to be able to detect at least four other viruses in this group. An ELISA test is only applicable to SJNNV from diseased larvae of striped jack.

F.6.4.2.3 Transmission Electron Microscopy (TEM) (Level III)

Virus particles are found in affected brain and retina by both TEM and negative staining. Positive stain TEM reveals non-enveloped, icosahedral, virus particles associated with vacuolated cells and inclusion bodies. The particles vary from 22-25 *nm* (European sea bass) to 34 *nm* (Japanese parrotfish) and form intracytoplasmic crystalline arrays, aggregates or single particles (both intra- and extracellular). In negative stain preparations, non-enveloped,

F.6 Viral Encephalopathy and Retinopathy (VER)

Species	Behaviour Changes	Appearance Changes	Onset of Clinical Signs
Barramundi	Uncoordinated darting and corkscrew swimming; off feed	Pale colouration, anorexia and wasting	Earliest onset at 9 days post-hatch. Usual onset at15-18 days post-hatch
European Sea Bass	Whirling swimming; off feed	Swim-bladder hyperinflation	Earliest onset at 10 days post-hatch. Usual onset 25-40 days post-hatch
Japanese Parrotfish	Spiral swimming	Darkened colour	First onset anywhere between 6-25 mm total length
Red-spotted Grouper	Whirling swimming	-	First onset at 14 days post-hatch (7-8 mm total length). Usual onset at 9-10 mm total length
Brown-spotted Grouper	-	-	20-50 mm total length
Striped Jack	Abnormal swimming	Swim-bladder hyperinflation	1-4 days post-hatch
Turbot	Spiral and/or looping swim pattern, belly-up at rest	Darkened colour	< 21 days post-hatch

Table F.6.4.1.1 – adapted from OIE (1997)

round to icosahedral particles, 25-30 *nm*, are detectable. These are consistent with VER nodaviruses.

F.6.4.2.4 Nucleic Acid Assay (Level III)

Reverse transcriptase PCR assays have been developed for VER nodavirus detection and identification.

F.6.5 **Modes of Transmission**

Vertical transmission of VER virus occurs in striped jack, and ovarian infection has been reported in European sea bass. Other modes of transmission have not been clearly demonstrated, but horizontal passage from juvenile fish held at the same site, and contamination of equipment cannot yet be ruled out. Experimental infections have been achieved in larval stripe jack and red-spotted grouper using immersion in water containing VER virus. Juvenile European sea bass have also been infected by inoculation with brain homogenates from infected individuals.

F.6.6 **Control Measures**

Control of VNN in striped jack and other affected species is complicated by the vertical transmission of the virus(es). Strict hygiene in hatcheries may assist in controlling VNN infection. Culling of detected carrier broodstock is one control option used for striped bass, however, there is some evidence that reduced handling at spawning can reduce ovarian infections and vertical transmission in some carrier fish. Control of clinical disease in striped bass using the following techniques has also shown some success:

- no recycling of culture water
- chemical disinfection of influent water and larval tanks between batches, and
- reduction of larval density from 15-30 larvae/litre to <15 larvae/litre (preferably fewer than 10 larvae/litre).

Anderson *et al.* (1993) reported that non-recycling of water, chemical sterilization of influent seawater and disinfection of half of the tanks during each hatching cycle was successful in a barramundi hatchery.

Extensive culture in ‘green ponds’ has also been related to low prevalences of clinical disease and/or histological lesions.

Arimoto *et al.* (1996) recommended the following measures: (a) disinfection of eggs (iodine or ozone) and materials (chlorine); (b) rearing of each batch of larvae/juveniles in separate tanks supplied with sterilized (UV or ozone) seawater; and (c) rigorous separation of larval and juvenile striped jack from brood fish.

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F.7 SPRING VIRAEMIA OF CARP (SVC)

F.7.1 **Background Information**

F.7.1.1 *Causative Agents*

Spring viraemia of carp (SVC) is caused by ssRNA Vesiculovirus (Rhabdoviridae), known as Spring viraemia of carp Virus (SVCV) or *Rhabdovirus carpio* (RVC) (Fijan 1999). More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

F.7.1.2 *Host Range*

SVCV infects several carp and cyprinid species, including common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idellus*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), crucian carp (*Carassius carassius*), goldfish (*C. auratus*), tench (*Tinca tinca*) and sheatfish (*Silurus glanis*).

F.7.1.3 *Geographic Distribution*

SVC is currently limited to the parts of continental Europe that experience low water temperatures over winter.

F.7.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999 – 2000)*

No reported case in any country during reporting period for 1999 and 2000 (OIE 1999, 2000b).

F.7.2 **Clinical Aspects**

Young carp and other susceptible cyprinids (F.7.1.2), up to 1 year old, are most severely affected. Overt infections are manifest in spring when water temperatures reach 11-17 °C. Poor physical condition of overwintering fish appears to be a significant contributing factor. Mortalities range from 30-70%.

Viral multiplication in the endothelial cells of blood capillaries, haematopoietic tissue and nephron cells, results in oedema and haemorrhage and impairs tissue osmoregulation. Kidney, spleen, gill and brain are the organs in which SVCV is most abundant during overt infection. Survivors demonstrate a strong protective immunity, associated with circulating antibodies, however, this results in a covert carrier state.

F.7.3 **Screening Methods**

More detailed information on methods for screening SVC can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

F.7.3.1 *Presumptive*

There are no methods for detection of sub-clinical infections using gross observations or routine histology.

F.7.3.1.1 Virology (Level III)

Screening for sub-clinical carriers uses tissue homogenates from the brain of any size fish or the ovarian fluids from suspect broodstock fish. Cell lines susceptible to SVCV are EPC and FHM. Any resultant CPE requires molecular-based assays as described under F.7.3.2.

F.7.3.2 *Confirmatory*

F.7.3.2.1 Immunoassays (Level III)

CPE products can be checked for SVCV using a virus neutralisation (VN) test, indirect fluorescent antibody tests (IFAT), and ELISA. IFAT can also be used on direct tissue preparations.

F.7.4 **Diagnostic Methods**

More detailed information on methods for diagnosis of SVC can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int
or selected references.

F.7.4.1 Presumptive

F.7.4.1.1 Gross Observations (Level I)

Sudden mortalities may occur with no other clinical signs. Behavioural clues are non-specific to SVC and include lethargy, separation from the shoal, gathering at water inlets or the sides of ponds and apparent loss of equilibrium.

External signs of infection are also non-specific, with fish showing varying degrees of abdominal distension (dropsy), protruding vents and trailing mucoid faecal casts. Haemorrhaging at the bases of the fins and vent, bulging eye(s) (pop-eye or exophthalmia), overall darkening and pale gills may also be present (Figs.F.7.4.1.1a, b, c and d).

Internal macroscopic signs of infection include an accumulation of body cavity fluids (ascites)

which may lead to the dropsy visible as abdominal distension, bloody and mucous-filled intestines, swim-bladder haemorrhage and gill degeneration.

F.7.4.1.2 Transmission Electron Microscopy (TEM) (Level III)

Detection of enveloped, bullet-shaped, viral particles measuring 90-180 nm in length and with a regular array of spicules on the surface in spleen, kidney and brain tissues, or in isolates from CPE in the cell-lines described under F.7.4.1.3, should be considered indicative of SVC in susceptible carp species showing other clinical signs of the disease. Viral replication takes place in the cytoplasm with maturation in association with the plasma membrane and Golgi vesicles.

F.7.4.1.3 Virology (Level III)

Whole fish (body length ≤4 cm), or viscera including kidney (fish 4 - 6 cm in length) or kidney, spleen and brain of larger fish, can be prepared for tissue culture using *Epithelioma papulosum cyprinae* (EPC) or FTM cell lines. Resultant CPE should be examined using the diagnostic techniques outlined below and under F.7.3.2.1 to confirm SVCV as the cause.

F.7.4.2 *Confirmatory*

F.7.4.2.1 Immunoassays (Level III)

As described under F.7.4.1.3, SVCV can be confirmed in CPE products using a virus neutralisation (VN) test, indirect fluorescent antibody tests (IFAT), and ELISA. IFAT can also be used on direct tissue preparations.

F.7.4.2.2 Nucleic Acid Assay (Level III)

RT-PCR techniques are under development.

F.7.5 **Modes of Transmission**

Horizontal transmission can be direct (contact with virus shed into the water by faeces, urine, reproductive fluids and, probably, skin mucous) or indirectly via vectors (fish-eating birds, the carp louse *Argulus foliaceus* or the leech *Piscicola geometra*). Vertical transmission is also possible via SVCV in the ovarian fluids (however, the rarity of SVC in fry and fingerling carp indicates that this may be a minor transmission pathway).

SVCV is hardy and can retain infectivity after exposure to mud at 4°C for 42 days, stream water at 10°C for 14 days, and after drying at 4-21°C for 21 days. This means that avenues for establishing and maintaining reservoirs of infection are relatively unrestricted. This, plus the broad direct and indirect mechanisms for transmission, makes this disease highly contagious and difficult to control.

F.7.6 **Control Measures**

No treatments are currently available although some vaccines have been developed. Most effort is applied to optimising the overwintering condition of the fish by reducing stocking density, reduced handling and strict maintenance of hygiene. New stocks are quarantined for at least two weeks before release into ponds for grow-out.

Control of spread means rapid removal and destruction of infected and contaminated fish immediately on detection of SVC. Repeat outbreaks may allow action based on presumptive diagnosis. First time outbreaks should undertake complete isolation of affected fish until SVC can be confirmed.

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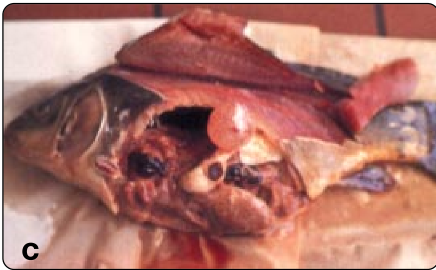
(EAFP)

Fig.F.8.4.1.1. Non-specific internal sign (petechial haemorrhage on muscle) of VHS infected fish.

(EAFP)



Figs.F.7.4.1.1a, b, c, d. Non-specific clinical signs of SVC infected fish, which may include swollen abdomen, haemorrhages on the skin, abdominal fat tissue, swim bladder and other.

F.8 VIRAL HAEMORRHAGIC SEPTICAEMIA (VHS)

F.8.1 **Background Information**

F.8.1.1 *Causative Agent*

Viral haemorrhagic septicaemia (VHS) is caused by ssRNA enveloped rhabdovirus, known as viral haemorrhagic septicaemia virus (VHSV). VHSV is synonymous with Egtved virus. Although previously considered to fall within the Lyssavirus genus (Rabies virus), the ICTV have removed it to "unassigned" status, pending evaluation of a proposed new genus – Novirhabdovirus – to include VHSV and IHNV (see http://www.ncbi.nlm.nih.gov/ICTV). Several strains of VHSV are recognised. More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

F.8.1.2 *Host Range*

VHS has been reported from rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), grayling (*Thymallus thymallus*), white fish (*Coregonus* spp.), pike (*Esox lucius*) and turbot (*Scophthalmus maximus*). Genetically distinct strains of VHSV have also been associated with disease in Pacific salmon (*Oncorhynchus* spp.), Pacific cod (*Gadus macrocephalus*) and Pacific herring (*Clupea pallasi*). These strains show little virulence in rainbow trout challenges (OIE 2000a). VHSV has also been isolated from Atlantic cod (*Gadus morhua*), European sea bass (*Dicentrarchus labrax*), haddock (*Melanogrammus aeglefinus*), rockling (*Rhinonemus cimbrius*), sprat (*Sprattus sprattus*), herring (*Clupea harengus*), Norway pout (*Trisopterus esmarkii*), blue whiting (*Micromesistius poutassou*), whiting (*Merlangius merlangius*) and lesser argentine (*Argentina sphyraena*) (Mortensen 1999), as well as turbot (*Scophthalmus maximus*) (Stone *et al.* 1997). Among each species, there is a high degree of variability in susceptibility with younger fish showing more overt pathology.

F.8.1.3 *Geographic Distribution*

VHSV is found in continental Europe, the Atlantic Ocean and Baltic Sea. Although VHSV-like infections are emerging in wild marine fish in North America, VHS continues to be considered a European-based disease, until the phylogenetic identities of the VHSV-like viruses which do not cause pathology in rainbow trout can be clearly established.

F.8.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

Japan reported the disease during second quarter of 2000, no other reports from other countries (OIE 1999, 2000b).

F.8.2 **Clinical Aspects**

The virus infects blood cells (leucocytes), the endothelial cells of the blood capillaries, haematopoietic cells of the spleen, heart, nephron cells of the kidney, parenchyma of the brain and the pillar cells of the gills. Spread of the virus causes haemorrhage and impairment of osmoregulation. This is particularly severe in juvenile fish, especially during periods when water temperatures ranging between 4 – 14°C.

F.8.3 **Screening Methods**

More detailed information on methods for screening VHS can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

F.8.3.1 *Presumptive*

F.8.3.1.1 Gross Observations (Level I) and Histopathology (Level II)

There are no gross visible clues (Level I) or histopathology clues (Level II) to allow presumptive diagnosis of sub-clinical VHS infections. Subclinical carriers should be suspected, however, in populations or stocks which originate from survivors of clinical infections or from confirmed carrier broodstock.

F.8.3.1.2 Virology (Level III)

VHSV can be isolated from sub-clinical fish on Bluegill Fry (BF-2), *Epithelioma papulosum cyprinae* (EPC) or rainbow trout gonad (RTG-2). Any resultant CPE requires further immunoassay or nucleic acid assay to confirm VHSV as the cause (F.8.3.2).

F.8.3.1.3 Immunoassay (Level III)

Immunohistochemistry can be used to highlight VHSV in histological tissue samples (which on their own cannot be used to screen sub-clinical infections). Due to the wide range of hosts and serotypes, however, any cross-reactions need to be confirmed via tissue culture and subsequent viral isolation as described under F.8.3.1.2.

F.8 Viral Haemorrhagic Septicaemia (VHS)

F.8.3.2 *Confirmatory*

F.8.3.2.1 Immunoassay (Level III)

Identification of VHSV from cell-line culture can be achieved using a virus neutralisation test, indirect fluorescent antibody test (IFAT) or ELISA.

F.8.3.2.2 Nucleic Acid Assay (Level III)

RT-PCR techniques have been developed.

F.8.4 **Diagnostic Procedures**

More detailed information on methods for diagnosis of VHS can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

F.8.4.1 *Presumptive*

F.8.4.1.1 Gross Observation (Level I)

There are no VHS-specific gross clinical signs. General signs are shared with bacterial septicaemias, IHN, osmotic stress, handling trauma, *etc.*, and include increased mortality, lethargy, separation from the shoal, gathering around the sides of ponds, nets or water inlets.

The skin may become darkened and haemorrhagic patches may be visible at the case of the fins, the vent and over the body surface. Gill may also be pale. Internal organ changes may or may not be present depending on the speed of onset of mortalities (stressed fish die quicker). Where present these include an accumulation of bloody body cavity fluids (ascites), mucous-filled intestines and pale rectal tissues. Pin-point haemorrhages may also be present throughout the muscle (Fig.F.8.4.1.1), fat (adipose) tissue and swim-bladder.

F.8.4.1.2 Virology (Level III)

VHSV can be isolated from whole alevin (body length ≤ 4 cm), viscera including kidney (fish 4 – 6 cm in length) or kidney, spleen and brain tissue samples from larger fish, using BF-2, EPC or RTG-2 (as described under F.8.3.1.2). Any resultant CPE requires further immunoassay or nucleic acid assay to confirm VHSV as the cause (F.8.3.2.1/2).

F.8.4.1.3 Immunoassay (Level III)

Immunohistochemistry can be used to highlight VHSV in histopathological lesions (however, histology is not a normal method of diagnosing VHS). Due to the wide range of hosts and serotypes, however, any cross-reactions need to be confirmed via tissue culture and subsequent viral isolation as described under F.8.3.1.2.

F.8.4.2 *Confirmatory*

As described under F.8.3.2.

F.8.5 **Modes of Transmission**

VHSV is shed in the faeces, urine and sexual fluids of clinically infected and sub-clinical carrier fish (wild and cultured). Once established at a site or in a water catchment system, the disease becomes enzootic because of the virus carrier fish. Water-borne VHSV can be carried 10-26 km downstream and remain infective. Mechanical transfer by fish-eating birds, transport equipment and non-disinfected eggs from infected broodstock, have all been demonstrated as viable routes of transmission (Olesen 1998).

F.8.6 **Control Measures**

No treatments are currently available, although DNA-based vaccines have shown some success under experimental conditions. Most control methods aim towards breaking the transmission cycle and exposure to carriers, as well as reducing stress. Pathogenic proliferation occurs at temperatures <15°C and periods of handling stress in sub-clinical populations.

Isolation, destruction and sterile/land-fill disposal of infected fish, as well as susceptible fish exposed downstream, along with disinfection of sites and equipment, has proven effective in controlling losses from this disease. Disinfection requires a minimum of 5 minutes contact with 3% formalin or 100 ppm iodine, 10 minutes with 2% sodium hydroxide and 20 minutes with 540 mg/L chlorine. Fallowing for at least 4 weeks when water temperatures exceed 15°C has also proven effective for re-stocking with VHSV-negative fish. These approaches have led to elimination of VHS from several areas in Europe.

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F.9 LYMPHOCYSTIS

F.9.1 Background Information

F.9.1.1 *Causative Agent*

Lymphocystis is caused by dsDNA, non-enveloped, iridoviruses, with particles measuring 200±50 *nm*, making them among the largest of the Iridoviridae. The iridovirus associated with gilthead sea bream (*Sparus aurata*) is known as Lymphocystis Disease Virus (LDV).

F.9.1.2 *Host Range*

Lymphocystis occurs in many marine and some freshwater fish families, including, herring (Clupeidae), smelt (Osmeridae), sea bass (Serranidae), flounder (Paralichthidae), snappers (Lutjanidae), perch (Percidae), drum (Sciaenidae), butterfly fishes (Chaetodontidae), cichlids (Cichlidae), gobies (Gobiidae) and sole (Soleidae).

F.9.1.3 *Geographic Distribution*

The geographic range of lymphocystis is probably global. The disease has been reported from Europe, North and Central America, Australia, Africa, Hawaii, the South Pacific and Asia.

F.9.2 Clinical Aspects

Lymphocystis is a common chronic and benign infection by an iridovirus that results in uniquely hypertrophied cells, typically in the skin and fins of fishes. The main clinical signs are white (occasionally pale red), paraffin-like nodules covering the skin and fins of sick fish (Fig. F.9.2a). Some particulate inclusions may be observed in the lymphoma lesion.

At maturity the lesions are irregularly elevated masses of pebbled texture. The colour is light cream to grayish, but covering epithelial tissue may be normally pigmented. Vascularity sometimes gives large clusters of cells a reddish hue. Considerable variation occurs in size, location, and distribution of the masses. Infected cells may also occur singly.

Although the infection is rarely associated with overt disease, mortalities can occur under culture conditions, possibly due to impaired gill, swimming or feeding capability with mechanically intrusive lesions. The primary effect, however, is economical, as fish with such grossly visible lesions are difficult to market.

F.9.3 Screening Methods

Currently there are no detection techniques that are sensitive enough to detect or isolate this group of iridoviruses from sub-clinically infected fish. To date, cell-culture techniques have been limited to isolation of virus from evident lymphoma lesions.

F.9.4 Diagnostic Methods

F.9.4.1 *Presumptive*

F.9.4.1.1 Gross Observations (Level I)

The main external signs associated with lymphocystis are white (or occasionally pale pink), paraffin wax-like nodules or growths over the skin and fins. Such growths may contain small granular-like particles, and some may show signs of vascularisation (extension of blood capillaries into the tissue growth) (Fig. F.9.4.1.1a and Fig. F.9.4.1.1b). The presence of the granular inclusions is an important for distinguishing lymphocystis from Carp Pox disease (caused by a *Herpesvirus*) (Fig.F.9.4.1.1c). The wax-like appearance is also an important feature which distinguishes Lymphocystis from fungal (mycotic) skin growths (Fig.F.9.4.1.1d).

F.9.4.2 *Confirmatory*

F.9.4.2.1 Histopathology (Level II)

Light microscopy of tissue sections of lymphoma reveal that the particulate inclusions are virus-induced giant cells of connective tissue origin, enveloped with a thick capsule. The diameter of each giant cell is about 500 Îm, a magnification of normal cell volume of 50,000 to 100,000-fold (Fig.F.9.4.2.1a). The distinctive capsule (Fig.F.9.4.2.1b), enlarged and centrally located nucleus and nucleolus, and cytoplasmic inclusions, are unique. No such cell alterations occur with Carp Pox *Herpesvirus* infections. In addition, there may be some eosinophilic, reticulate (branching) inclusion bodies in cytoplasm of the giant cell. These correspond to the viral replicating bodies which have a light refractive density which renders them "cytoplasmic-like" and demonstrate one of the few instances when a viral aetiology can be diagnosed at the light microscope level with a high degree of confidence. Identification of the exact virus(es) involved requires further investigation, however, this level of diagnosis is sufficient to allow control advice to be made (F.9.6).

F.9 Lymphocystis

(MG Bondad-Reantaso)

Fig.F.9.2a. Wild snakehead infected with lymphocystis showing irregularly elevated masses of pebbled structure.

(J Yulin)

Fig.F.9.4.1.1a. Flounder with severe lymphocystis.

(J Yulin)

Fig.F.9.4.1.1b. Lymphocystis lesions showing granular particle inclusions.

(J Yulin)

Fig.F.9.4.1.1c. Carp Pox Disease caused by Herpesvirus.

(J Yulin)

Fig.F.9.4.1.1d. Goldfish with fungal (mycotic) skin lesions

(J Yulin)

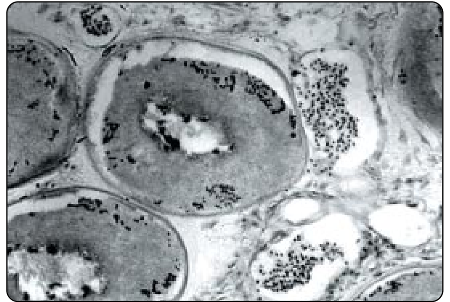

Fig.F.9.4.2.1a. Giant (hypertrophied lymphoma cells with reticulate or branching inclusion bodies around the nuclei.

(J Yulin)

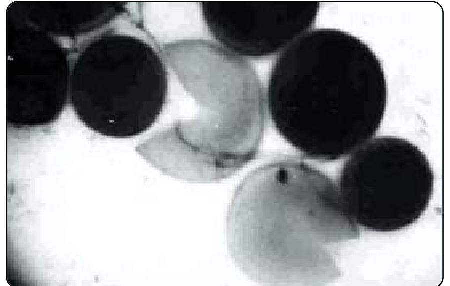

Fig.F.9.4.2.1b. Impression smear of lymphocystis showing some giant cells, and hgaline capsules (membrane).

F.9.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

TEM of ultra-thin section of lymphocystis tissue is the principal method of further confirming gross and light microscope observations. The viral particles show up as large icosahedral (roughly hexagonal-spherical) particles measuring 150-300 *nm* within the encapsulated cell cytoplasm. Ultrastructural features of lymphocystis iridovirus particles include a dense core within two unit membranes making up the capsid (Fig.F.9.4.2.2a and Fig.F.9.4.2.2b). Note the difference from *Herpesvirus* in Carp Pox, which are enveloped and smaller virions (Fig.F.9.4.2.2c).

(J Yulin)

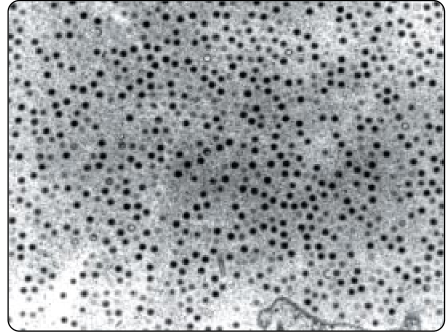

Fig.F.9.4.2.2a. Electron micrograph showing numerous viral particles in cytoplasm.

F.9.4.2.3 Virology (Level III)

Due to the relative ease of finding and identifying the viruses associated with lymphocystis lesions (compared with the viral agents of other fish diseases), there has been little emphasis on cell culture as a means of confirming diagnosis of the disease. However, the growing impact of this disease in aquaculture situations around the world has increased interest in differentiating between the iridoviral agents involved and enhancing apparent acquired immunity to infection. A new cell-line from gilt-head sea bream is currently under investigation and has shown promise for isolating lymphocystis iridoviruses.

(J Yulin)

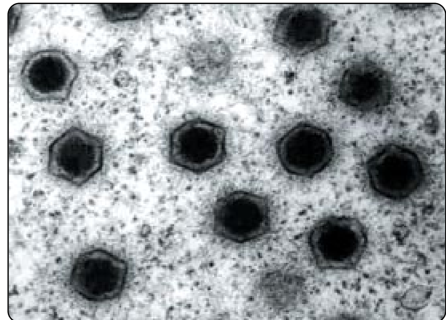

Fig.F.9.4.2.2b. Enlarged viral particles showing typical morphology of iridovirus (100 mm = bar).

F.9.5 Modes of Transmission

Horizontal contact and water-borne transmission appear to be the principal mechanism for lymphocystis virus spread. This is reinforced by proliferation of the problem under intensive culture conditions. High population density and external trauma enhance transmission. External surfaces including the gills appear to be the chief portal of epidermal entry. The oral route seems not to be involved, and there is no evidence of vertical transmission.

(J Yulin)

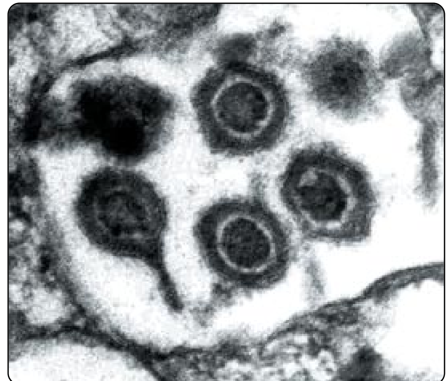

Fig.F.9.4.2.2c. *Herpervirus* in Carp Pox showing enveloped and smaller virions compared to lymphocystis virus.

F.9.6 Control Methods

At present, there is no known method of therapy or of immunization. There is some evidence of antibodies in at least one flatfish species, however, this remains to be investigated further. Avoidance of stocking with clinically infected fish, early detection through monitoring and sterile (land-fill or chemical) disposal, along with minimising stocking densities and handling skin-trauma, have proven to be effective controls.

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BACTERIAL DISEASE OF FINFISH
F.10 BACTERIAL KIDNEY DISEASE (BKD)

F.10.1 **Background Information**

F.10.1.1 *Causative Agent*

Bacterial kidney disease (BKD) is caused by *Renibacterium salmoninarum*, a coryneform, rod-shaped, Gram-positive bacterium that is the sole species belonging to the genus *Renibacterium*. More detailed information about the diseases can be found in the OIE Manual for Aquatic Animal Diseases (OIE 2000a).

F.10.1.2 *Host Range*

Fish of the Salmonidae family are clinically susceptible, in particular the *Oncorhynchus* species (Pacific salmon and rainbow trout).

F.10.1.3 *Geographic Distribution*

BKD occurs in North America, Japan, Western Europe and Chile.

F.10.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

Japan reported BKD occurrence for whole year except for the month of December for both 1999 and 2000 reporting period. Pakistan suspected the disease from July to December of 1999 (OIE 1999, 2000b).

F.10.2 **Clinical Aspects**

Renibacterium salmoninarum infections can build up over a long period of time, with clinical disease only appearing in advanced infections, usually when the fish have completed their first year of life. Virulence of *R. salmoninarum* varies with:

- the strain of bacterium
- the salmon species infected
- environmental and holding conditions.

The bacteria can evade lysosomal breakdown by the blood cells that engulf them, thus avoid destruction by the fishes' primary defence mechanism. Nutrition and seawater transfers can also affect the pathogenicity of *R. salmoninarum* infections and broodstock infection levels are believed to have a direct correlation to susceptibility in their offspring. Progeny of parent stock with low levels or no infection with *R. salmoninarum* show better survival than offspring from BKD compromised fish. This may reflect greater transmission titres by the latter (F.10.5).

F.10.3 **Screening Methods**

Detailed information on methods for screening BKD can be found at the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or selected references.

F.10.3.1 *Presumptive*

F.10.3.1.1 Gross Observations (Level I) and Histopathology (Level II)

There are no gross signs or histological lesions that can be detected in sub-clinical carriers of *Renibacterium salmoninarum*.

F.10.3.1.2 Bacteriology (Level II)

When no lesions are present, the kidney should be selected for culture. In mature females, coelomic fluids may also be used. Specialised growth media, such as, kidney disease medium enriched with serum (KDM2) or charcoal (KDMC), or selective kidney disease medium (SKDM) are required due to the fastidious nature of *Renibacterium salmoninarum*.

Growth requires 2-3 weeks, but may take up to 12 weeks. Colonies are pinpoint to 2 mm in diameter, white-creamy, shiny, smooth, raised and entire (Fig.F.10.3.1.2a). The rods (Fig.F.10.3.1.2b) are 0.3-1.5 x 0.1-1.0 mm, Gram-positive, PAS-positive, non-motile, non acid-fast, frequently arranged in pairs or chains or in pleiomorphic forms ("Chinese letters"). Old cultures may achieve a granular or crystalline appearance. Transverse sections through such colonies will reveal the presence of Gram-positive rods in a crystalline matrix. Although few other bacteria have these growth characteristics, identification of the bacteria should be confirmed by immunoassay (F.10.3.2.1) or nucleic acid assay (F.10.3.2.2).

F.10.3.2 *Confirmatory*

F.10.3.2.1 Immunoassays (Level II/III)

Agglutination tests, direct and indirect fluorescent antibody tests (DFAT, IFAT) and ELISA kits are now available that can be used to detect *R. salmoninarum* antigen in fish tissues, as well as from bacterial cultures. The ELISA tests are believed to be the most sensitive to low-titre infections, hence they are recommended for screening for sub-clinical carriers (such as ovarian fluids from broodstock salmonids). Commercially produced kits are

(M Yoshimizu)

Fig.F.10.3.1.2a. Pinpoint colonies up to 2 mm in diameter of *Renibacteriium salmonimarum,* white-creamy, shiny, smooth, raised and entire; three weeks after incubation at 15°C on KDM-2 medium.

(M Yoshimizu)

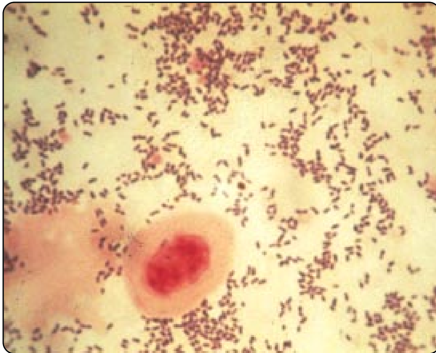

Fig.F.10.3.1.2b. Renibacterium salmoninarum rods, isolated from masou salmon.

(M Yoshimizu)

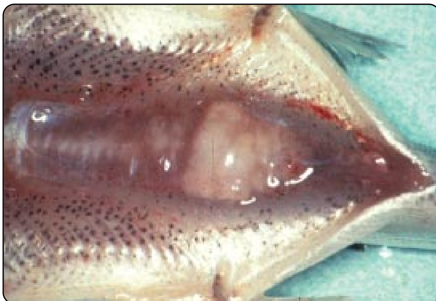

Fig.F.10.4.1.1a. Kidney of masou salmon showing swelling with irregular grayish.

also available, which contain specific instructions. Positive ELISA results, using either polyclonal or monoclonal antibodies, should be corroborated with other diagnostic tests, especially for sub-clinical cases, or first time isolations (Griffiths *et al*. 1996).

(EAFP)

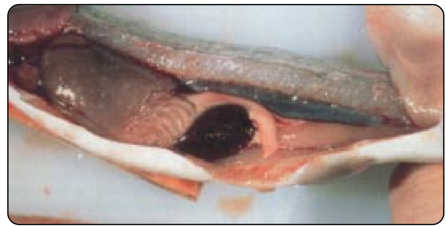

Fig.F.10.4.1.1b. Enlargement of spleen is also observed from BKD infected fish.

F.10.3.2.2 Nucleic Acid Assays (Level III)

Renibacterium salmoninarum primers have been developed for PCR-probes. These can detect *R. salmoninarum* DNA in tissue homogenates. The primers have been published and some kits are now commercially available.

F.10.4 **Diagnostic Methods**

Detailed information on methods for diagnosis of BKD can be found at the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or selected references.

F.10.4.1 *Presumptive*

F.10.4.1.1 Gross Observations (Level I)

Gross clinical signs are not usually evident until infections have become well-advanced (usually after at least 1 year). These include exophthalmia (pop-eye), varying degrees of abdominal distension (dropsy) due to disruption of the kidney excretory function, skin lesions and haemorrhaging.

Internally, there is evidence of grey/white lesions (granulomas) in all the organs, but especially the kidney (Fig.F.10.4.1.1a); enlargement of spleen (Fig.F.10.4.1.1b) is also observed. The greyish spots may show signs of multiplication and coalescence until the whole kidney appears swollen and bloated with irregular greyish patches. BKD can be distinguished from **proliferative kidney disease (PKD)** in salmonids, where the kidney becomes enlarged but there is no associated grey discolouration. Another salmonid kidney disease – **nephrocalcinosis** – only affects the urinary

ducts, which develop a white porcelain texture and colour.

F.10.4.1.2 Smears (Level I)

Smears from tissue lesions of susceptible hosts stained with Gram's stain or other metachromatic stain may reveal large numbers of small Gram-positive, rod-shaped, bacteria. Care should be taken not to confuse these with the melanin granules commonly present in kidney tissues. Other Gram-positive bacteria, such as *Lactic* species, may also be present, so further bacteriological identification methods are required.

F.10.4.1.3 Bacteriology (Level II)

Whenever possible, culturing should be used for confirmation despite the difficulties imposed by the slow, fastidious growth of *Renibacterium salmoninarum*. Presumptive diagnosis is also possible from bacterial culture due its slow growth (2-3 weeks) at 15°C. Kidney and other organs with suspicious lesions should be sampled. Protocols for culture are as described under F.10.3.1.2. Although few other bacteria have these growth characteristics, identification of the bacteria should be confirmed by immunoassay (F.10.3.2.1) or nucleic acid assay (F.10.3.2.2)

F.10.4.2 *Confirmatory*

F.10.4.2.1 Immunoassay (Level II/III)

Slide agglutination tests can be used for rapid identification of culture colonies. Bacterial agglutination is determined by comparison with duplicate suspension containing rabbit serum, as a control. Co-agglutination with *Staphylococcus aureus* (Cowan I strain) sensitised with specific immunoglobulins is also effective at enhancing the agglutination process (Kimura and Yoshimizu 1981).

For immunofluorescence (direct and indirect) and ELISA tests, MAbs against specific determinants are recommended to avoid cross-reactions with other bacteria. As noted under F.10.3.2.1, positive results, using either polyclonal or monoclonal antibodies, should be corroborated with other diagnostic tests, especially for first time isolations (Griffiths *et al*. 1996).

F.10.4.2.2 Nucleic Acid Assays (Level III)

As described under F.10.3.2.2, *Renibacterium salmoninarum* PCR-probes are now available. Cross-checking positive samples with other diagnostic methods (bacteriology, immunoassay), however, is highly recommended, especially for first time isolations (Hiney and Smith 1999).

F.10.5 **Modes of Transmission**

Renibacterium salmoninarum is widely distributed in both freshwater and marine environments. It can be transmitted horizontally by water-borne release and faecal contamination, as well as via reservoir hosts which span all salinity ranges. Indirect vertical transmission via reproductive fluids and spawning products is also possible for sub-clinical carriers of the bacteria.

F.10.6 **Control Measures**

Due to its intracellular location in the host fish, BKD is difficult to treat with antibiotics. Injection of female broodstock with erythromycin at regular intervals prior to spawning appears to have some success in preventing vertical transmission to eggs. Vaccination and medicated feeds have also shown some success in reducing the occurrence of BKD, however, results have varied with strain of *R. salmoninarum* and host species.

Most emphasis is placed on breaking vertical and horizontal transmission routes (F.10.5). Culling of high BKD-titre broodstock, reducing stocking density, avoiding contact with sub-clinical carriers/reservoirs, reducing handling stress and avoiding unacclimatised transfer from fresh to saltwater, have all proven effective in reducing BKD pathogenicity.

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FUNGUS ASSOCIATED DISEASE
F.11 EPIZOOTIC ULCERATIVE SYNDROME (EUS)

F.11.1 Background Information

F.11.1.1 *Causative Factors*

The mycotic granulomas in EUS-affected tissues are caused by the Oomycete fungus *Aphanomyces invadans* (also known as *A. invaderis, A. piscicida*, Mycotic Granuloma-fungus (MG) and ERA [EUS-related *Aphanomyces*]). It is also known as Red spot disease (RSD). More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

F.11.1.2 *Host Range*

EUS affects freshwater and estuarine warm water fish and was first reported in farmed ayu (*Plecoglossus altivelis*) in Japan (Fig.F.11.1.2a). Severe outbreaks occurred in Eastern Australia affecting estuarine fish, particularly grey mullet (*Mugil cephalus*). Region-wide, over 50 species (Fig.F.11.1.2b) have been confirmed affected by histopathological diagnosis (Lilley *et al.*, 1998), but some important culture species including tilapia, milkfish and Chinese carps have been shown to be resistant.

F.11.1.3 *Geographic Distribution*

EUS was first reported in Japan and subsequently in Australia. Outbreaks have shown a westward pattern of spread through Southeast and South Asia. EUS has also spread westward with major outbreaks reported in Papua New Guinea, Malaysia, Indonesia, Thailand, Philippines, Sri Lanka, Bangladesh and India. EUS has most recently been confirmed in Pakistan. The pathology demonstrated by ulcerative mycosis (UM)-affected estuarine fish along the Atlantic coast of USA is indistinguishable from EUS, but further work is required to compare the causal agents involved in each case.

F.11.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

Australia, Bangladesh, India, Japan, Lao PDR, Nepal, Philippines, Sri Lanka, and Thailand reported the disease on various months for the reporting year 1999; for the year 2000, Australia, Bangladesh, India, Japan, Lao PDR, Nepal, Pakistan, Philippines and Thailand reported positive occurrence of EUS (OIE 1999, OIE 2000b).

F.11.2 Clinical Aspects

Affected fish typically show necrotic dermal ulcers, characterised histologically by the presence of distinctive mycotic granulomas in underlying tissues. The mycotic granulomas in EUS-affected tissues are caused by the Oomycete fungus *Aphanomyces invadans*. Initial lesions may appear as red spots (Fig.F.11.2a), which become deeper as the infection progresses and penetrate underlying musculature (Fig.F.11.2b). Some advanced lesions may have a raised whitish border. High mortalities are usually associated with EUS outbreaks but, in certain cases, where fish do not succumb to secondary invasion of these gaping wounds, ulcers may be resolved.

F.11.3 Screening Methods

There are no screening methods for sub-clinical animals available.

F.11.4 Diagnostic Methods

More detailed information on methods for diagnosis of EUS can be found in the OIE Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or selected references..

F.11.4.1 *Presumptive*

F.11.4.1.1 Gross Observations (Level I)

The gross appearance of lesions varies between species, habitat and stage of lesion development (Fig.F.11.4.1.1a). The most distinctive EUS lesion is the open dermal ulcer. However, other diseases may also result in similar clinical lesions (Fig.11.4.1.1b) and it is, therefore, important to confirm the presence of *A. invadans* to ensure accurate diagnosis.

F.11.4.1.2 Rapid Squash Muscle Preparation (Level I)

Presumptive diagnosis of EUS in susceptible fish showing dermal lesions can be made by demonstrating aseptate hyphae (12-30 ?m in diameter) in squash preparations of the muscle underlying the visible lesion (Fig.11.4.1.2). This can be achieved using a thin piece of muscle squashed between two glass plates or microscope slides, examined using a light or dissecting microscope under field conditions.

F.11 Epizootic Ulcerative Syndrome (EUS)

(K Hatai)

Fig.F.11.1.2a. Ayu, *Plecoglatus altivelis,* infected with mycotic granulomatosis.

(RB Callinan)

Fig.F.11.1.2b. EUS affected farmed silver perch *Bidyanus bidyanus* from Eastern Australia.

(MG Bondad-Reantaso)

Fig.F.11.2a. Catfish showing initial EUS red spots.

(MG Bondad-Reantaso)

Fig.F.11.4.1.1a. Wild mullet in Philippines (1989) with EUS.

(MG Bondad-Reantaso)

Fig.F.11.2b. Snakehead in Philippines (1985) showing typical EUS lesions.

(MG Bondad-Reantaso)

Fig.F.11.4.1.1b. Red spot disease of grass carp in Vietnam showing ulcerative lesions.

F.11.4.2 *Confirmatory*

F.11.4.2.1 Histopathology (Level II)

Confirmatory diagnosis requires histological demonstration of typical granulomas and invasive hyphae using haematoxylin and eosin (Fig.F.11.4.2.1a) or a general fungus stain (*e.g.* Grocott's) (Fig.F.11.4.2.1b). Early EUS lesions show shallow haemorrhagic dermatitis with no obvious fungal involvement. Later lesions demonstrate *A. invadans* hyphae penetrating the skeletal muscle tissues and increasing inflammation. The fungus elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae, a typical characteristic of EUS. The lesion progresses from a mild chronic dermatitis, to a severe, locally pervasive, necrotising dermatitis, with severe degeneration of the muscle. The most typical lesions are large, open, haemorrhagic ulcers about 1-4 cm in diameter. These commonly show secondary infections with bacteria, and pathogenic strains of *Aeromonas hydrophila* have been isolated from lesions.

F.11.4.2.2 Mycology (Level II)

Moderate, pale, raised, dermal lesions are most suitable for fungal isolation attempts. Remove

the scales around the periphery of the lesion and sear the underlying skin with a red-hot spatula to sterilise the surface. Using a sterile scalpel blade and sterile, fine pointed, forceps, cut through skin underlying the seared area and cut horizontally to lift the superficial tissues and expose the underlying muscle. Ensure the instruments do not contact the external surface and contaminate the underlying muscle. Aseptically excise 2 mm^3 pieces of muscle, approximately 2 mm^3, and place on a Petri dish containing Czapek Dox agar with penicillin G (100 units/ml) and oxolinic acid (100 mg/ml). Seal plates and incubate at room temperature examining daily. Transfer emerging hyphal tips onto fresh plates of Czapek Dox agar until cultures are free of contamination.

The fungus can be identified to genus by inducing sporogenesis (Fig.F.11.4.2.2a) and demonstrating the asexual characteristics of *Aphanomyces* as described in Lilley *et al.* (1998). *A. invadans* is characteristically slow growing in culture (Fig.F.11.4.2.2b) and fails to grow at 37°C on GPY agar (GP broth with 0.5 g/l yeast extract and 12 g/l technical agar). Detailed temperature-growth profiles are given in Lilley and Roberts (1997). Confirmation that the isolate is *A. invadans* can be made by injecting a 0.1 ml suspension of 100+ motile zoospores intramuscularly in EUS-susceptible fish (preferably *Channa striata*) at 20°C, and demonstrating histologically growth of aseptate hyphae 12-30 μm in diameter in muscle of fish sampled after 7 days, and typical mycotic granulomas in muscle of fish sampled after 14 days.

F.11.5 **Modes of Transmission**

The spread of EUS is thought to be due to flooding and movement of affected and/or carrier fish. *Aphanomyces invadans* is considered to be the **"necessary cause"** of EUS, and is present in all cases, however, an initial skin lesion is required for the fungus to attach and invade underlying tissues. This lesion may be induced by biotic or abiotic factors. In Australia and Philippines, outbreaks have been associated with acidified water (due to acid sulfate soil runoff), along with low temperatures, presence of susceptible fish and *A. invadans* propagules. In other areas, where acid water does not occur, it is possible that other biological (*e.g.*, rhabdovirus infection) or environmental factors (*e.g.*, temperature) may initiate lesions.

F.11.6 **Control Measures**

Control in wild populations is impossible in most cases. Selection of resistant species for culture purposes currently appears to be the most effective means of farm-level control. Where changing culture species is not an option, measures should be taken to eradicate or exclude the fungus through:

- drying and liming of ponds prior to stocking
- exclusion of wild fish
- use of prophylactically-treated, hatchery-reared fry
- use of well-water
- salt bath treatments
- disinfection of contaminated nets and equipment.

F.11.7 **Selected References**

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F.11 Epizootic Ulcerative Syndrome (EUS)

(MG Bondad-Reantaso)

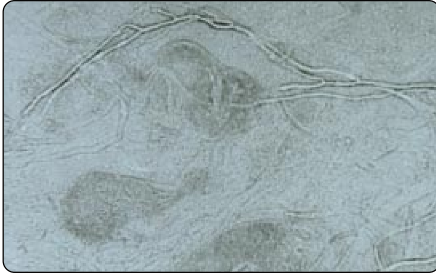

Fig.F.11.4.1.2. Granuloma from squash preparation of muscle of EUS fish.

(MG Bondad-Reantaso)

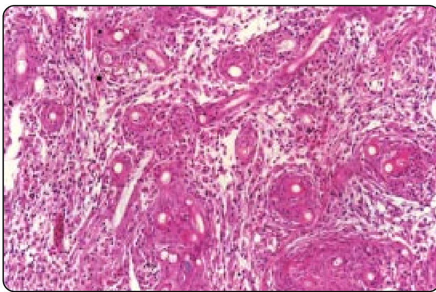

Fig.F.11.4.2.1a. Typical severe mycotic granulomas from muscle section of EUS fish (H & E).

(MG Bondad-Reantaso)

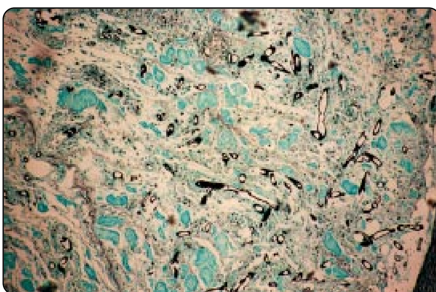

Fig.F.11.4.2.1b. Mycotic granulomas showing fungal hyphae (stained black) using Grocotts stain.

(K Hatai)

Fig.F.11.4.2.2a. Typical characteristic of *Aphanomyces* sporangium.

(MG Bondad-Reantaso)

Fig.F.11.4.2.2b. Growth of *Aphanomyces invadans* on GP agar.

Chinabut, S. and R.J. Roberts. 1999. Pathology and Histopathology of EpizooticUlcerative Syndrome (EUS). Aquatic Animal Health Research Institute,Department of Fisheries, Royal Thai Government, Bangkok, Thailand, 33p. ISBN 974-7604-55-8.

Fraser, G.C., R.B. Callinan, and L.M. Calder. 1992. *Aphanomyces* species associatedwith red spot disease: an ulcerative disease of estuarine fish from eastern Australia. *J. Fish Dis.* 15:173-181.

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Lilley, J.H., Callinan, R.B., Chinabut, S., Kanchanakhan, S., MacRae, I.H., and Phillips,M.J. 1998. Epizootic Ulcerative Syndrome (EUS) Technical Handbook. TheAquatic Animal Health Research Institute, Bangkok. 88p.

Lilley, J.H. and Roberts, R.J. 1997. Pathogenicity and culture studies comparing Aphanomyces involved in epizootic ulcerative syndrome (EUS) with other similar fungi. *J. Fish Dis.* 20: 135-144.

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Roberts, R.J., B. Campbell and I.H. MacRae (eds). 1994. Proceedings of the Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January 1994. The Aquatic Animal Health Research Institute. Bangkok, Thailand.

Tonguthai, K. 1985. A preliminary account of ulcerative fish diseases in the Indo-Pacific region (a comprehensive study based on Thai experiences). National Inland Fisheries Institute, Bangkok, Thailand. 39p.

ANNEX F.AI. OIE REFERENCE LABORATORIES FOR FINFISH DISEASES

Disease	Expert/Laboratory
Epizootic haematopoietic necrosis virus (EHNV)	**Dr. A. Hyatt** Australian Animal Health Laboratory Geelong, Victoria 3213, AUSTRALIA Tel: 61-3-52275000 Fax: 61-3-52275555 E-mail: alex.hyatt@dah.csiro.au
	Dr. R. Whittington Elizabeth MacArthur Agricultural Institute PMB 8, Camden NSW 2570, AUSTRALIA Tel: 61-2-46293333 Fax: 61-2-46293343 E-mail: Richard.Whittington@smtpgwy.agric.nsw.gov.au
Infectious haematopoietic necrosis virus (Rhabdoviruses)	**Dr. J. A. Leong** Oregon State University Department of Microbiology Nash Hall 220, Corvallis, Oregon 93331-3804 UNITED STATES of AMERICA Tel: 1-541-7371834 Fax: 1-541-7370496 E-mail: leongj@orst.edu
	Dr. J. Winton Western Fisheries Research Center 6505 N.E. 65th Street Seattle, Washington 98115 UNITED STATES of AMERICA E-mail: jim_winton@nbs.gov
Onchorhynchus masou virus	**Dr. M. Yoshimizu** Laboratory of Microbiology Faculty of Fisheries Hokkaido University 3-1-1, Minato-cho, Hakodate Hokkaido 041-0821 JAPAN Tel./Fax: 81-138-408810 E-mail: yosimizu@pop.fish.hokudai.ac.jp
Spring viremia of carp virus	**Dr. B.J. Hill** The Centre for Environment, Fisheries and Aquaculture Sciences (CEFAS) Barack Road, the Nothe, Weymouth, Dorset DT4 8UB UNITED KINGDOM Tel: 44-1305-206626 Fax:44-1305-206627 E-mail: b.j.hill@cefas.co.uk
Viral haemorrhagic septicaemia virus	**Dr. N.J. Ollesen** Danish Veterinary Laboratory Hangovej 2, DK-8200 Aarhus N DENMARK Tel: 45-89372431 Fax:45-89372470 E-mail: njo@svs.dk

Annex F.AI. OIE Reference Laboratories for Finfish Diseases

Channel catfish virus	**Dr. L.A. Hanson** Fish Diagnostic Laboratory College of Veterinary Medicine Mississippi State University Box 9825, Spring Street Mississippi 39762 UNITED STATES of AMERICA Tel: 1-662-3251202 Fax: 1-662-3251031 E-mail: hanson@cvm.msstate.edu
Viral encephalopathy and retinopathy	**Dr. G. Bovo** Instituto Zooprofilaticco Sperimentale delle Venezie Dipartimento di Ittiopatologia, Via Romea 14/A 35020 Legnaro PD ITALY Tel: 39-049-8830380 Fax: 39-049-8830046 E-mail: bovo.izs@interbusiness.it
	Dr. T. Nakai Fish Pathology Laboratory Faculty of Applied Biological Sciences Hiroshima University Higashihiroshima 739-8528 JAPAN Tel: 81-824-247947 Fax: 81-824-227059 E-mail: nakaitt@ipc.hiroshima-u.ac.jp
Infectious pancreatic necrosis	**Dr. B.J. Hill** The Centre for Environment, Fisheries and Aquaculture Sciences (CEFAS) Barack Road, the Nothe, Weymouth, Dorset DT4 8UB UNITED KINGDOM Tel: 44-1305-206626 Fax:44-1305-206627 E-mail: b.j.hill@cefas.co.uk
Infectious salmon anaemia	**Dr. B. Dannevig** National Veterinary Institute Ullevalsveien 68 P.O. Box 8156 Dep., 0033 Oslo NORWAY Tel: 47-22-964663 Fax: 47-22-600981 E-mail: birgit.dannevig@vetinst.no
Epizootic ulcerative syndrome	**Dr. Kamonporn Tonguthai** Aquatic Animal Health Research Institute Department of Fisheries Kasetsart University Campus Jatujak, Ladyao, Bangkok 10900 THAILAND Tel: 662-5794122 Fax: 662-5613993 E-mail: kamonpot@fisheries.go.th
Bacterial kidney disease	**Dr. R.J. Pascho** Western Fisheries Research Center U.S. Geological Survey Biological Resources Division 6505 N.E. 65th Street Seattle, Washington 98115

	UNITED STATES of AMERICA Tel: 1-206-5266282 Fax: 1-206-5266654 E-mail: ron_pascho@usgs.gov
Enteric septicaemia of catfish	**Dr. L.A. Hanson** Fish Diagnostic Laboratory College of Veterinary Medicine Mississippi State University Box 9825, Spring Street Mississippi 39762 UNITED STATES of AMERICA Tel: 1-662-3251202 Fax: 1-662-3251031 E-mail: hanson@cvm.msstate.edu
Piscirikettsiosis	**Dr. J.L. Fryer** Distinguished Professor Emeritus Department of Biology 220 Nash Hall Oregon State University Corvallis, Oregon 97331-3804 Tel: 1-541-7374753 Fax:1-541-7372166 E-mail: fryerj@bcc.orst.edu
Gyrodactylosis (*Gyrodactylus salaris*)	**Dr. T. Atle Mo** National Veterinary Institute Ullevalsvein 68 P.O. Box 8156 Dep., 0033 Oslo NORWAY Tel: 47-22-964722 Fax: 47-22-463877 E-mail: tor-atle.mo@vetinst.no
Red sea bream iridoviral disease	**Dr. K. Nakajima** Virology Section, Fish Pathology Division National Research Institute of Aquaculture Fisheries Agency 422-1 Nakatsuhama, Nansei-cho Watarai-gun Mie 516-0913 JAPAN Tel: 81-599661830 Fax: 81-599661962 E-mail: kazuhiro@nria.affrc.go.jp

ANNEX F.AII. LIST OF REGIONAL RESOURCE EXPERTS FOR FINFISH DISEASES IN ASIA PACIFIC[1]

Disease	Expert
Epizootic ulcerative syndrome (EUS)	**Dr. Richard Callinan** NSW Fisheries, Regional Veterinary Laboratory Wollongbar NSW 2477 **AUSTRALIA** Tel (61) 2 6626 1294 Mob 0427492027 Fax (61) 2 6626 1276 E-mail: richard.callinan@agric.nsw.gov.au
	Dr. C.V. Mohan Department of Aquaculture College of Fisheries, UAS Mangalore-575002 **INDIA** Tel: 91 824 439256 (College); 434356 (Dept), 439412 (Res) Fax: 91 824 438366 E-mail: cv_mohan@yahoo.com
	Prof. Kishio Hatai Divison of Fish Diseases Nippon Veterinary and Animal Science University 1-7-1 Kyonan-cho, Musashino, Tokyo 180 **JAPAN** Tel: 81-0422-31-4151 Fax: 81-0422-33-2094 E-mail: hatai@scan-net.ne.jp
	Ms. Susan Lumanlan-Mayo Fish Health Section Bureau of Fisheries and Aquatic Resources Arcadia Building, 860 Quezon Avenue Quezon City, Metro Manila **PHILIPPINES** Tel/Fax: 632-372-5055 E-mail: slmayo99@yahoo.com
	Mr. Jose O. Paclibare Fish Health Section Bureau of Fisheries and Aquatic Resources Arcadia Building, 860 Quezon Avenue Quezon City, Metro Manila **PHILIPPINES** Tel/Fax: 632-372-5055 E-mail: jopac@edsamail.com.ph
	Dr. Erlinda Lacierda Fish Health Section Aquaculture Department Southeast Asian Fisheries Development Center Tigbauan, Iloilo 5021 **PHILIPPINES** Tel: 63 33 335 1009 Fax: 63 33 335 1008 E-mail: eclacier@aqd.seafdec.org.ph
	Dr. Somkiat Kanchanakhan Aquatic Animal Health Research Institute Department of Fisheries Kasetsart University Campus

[1] The experts included in this list have previously been consulted and agreed to provide valuable information and health advise concerning their particular expertise.

	Jatujak, Ladyao, Bangkok 10900 **THAILAND** Tel: 662-5794122 Fax: 662-5613993 E-mail: somkiatkc@fisheries.go.th
	Dr. Supranee Chinabut Aquatic Animal Health Research Institute Department of Fisheries Kasetsart University Campus Jatujak, Ladyao, Bangkok 10900 **THAILAND** Tel: 662-5794122 Fax: 662-5613993 E-mail: supranee@fisheries.go.th
	Dr. Melba B. Reantaso Network of Aquaculture Centres in Asia Pacific Department of Fisheries Compound Kasetsart University Campus Jatujak, Ladyao, Bangkok 10900 **THAILAND** Tel: 662- 561-1728 to 9 ext. 113 Fax: 662-561-1727 E-mail: Melba.Reantaso@enaca.org
Viral nervous necrosis (VNN) Viral encephalopathy and retinopathy (VER)	**Dr. Kei Yuasa** Fisheries and Aquaculture International Co., Ltd. No. 7 Khoji-machi Bldg., Room B105 4-5 Khoji-machi, Chiyoda-ku Tokyo 102-0083 **JAPAN** Tel: 81-3-3234-8847 Fax:81-3-3239-8695 E-mail: fai@faiaqua.com; yuasakei@hotmail.com
	Dr Myoung-Ae Park Pathology Division National Fisheries Research and Development Institute South Sea Regional Fisheries Research Institute 347 Anpo-ri, Hwayang-myun, Yeosu-City Chullanam-do, 556-820 **KOREA RO** Tel: 82-662-690-8989 Fax: 82-662-685-9073 E-mail : mapark@haema.nfrda.re.kr
	Dr. Lin Li Guangdong Daya Wan Fisheries Development Center Aotou Town Huizhou City, Guangdong Province **PEOPLE's REPUBLIC OF CHINA** Tel: 86-752-5574225 Fax: 86-752-5578672 E-mail: hzgdfdc@pub.huizhou.gd.cn; linli39@hotmail.com
	Dr. Qiwei Qin Tropical Marine Science Institute National University of Singapore 10 Kent Ridge Crescent 119260 **SINGAPORE** Tel: +65-7749656 Fax: +65-7749654 Email: tmsqinqw@nus.edu.sg

Annex F.AII.List of Regional Resource Experts for Finfish Diseases in Asia Pacific

	Dr Shau Chi Chi Department of Zoology National Taiwan University **TAIWAN PROVINCE of CHINA** Fax : 886 2 2367 3852 E-mail : shauchi@ccms.ntu.edu.tw
	Dr Nguyen Huu Dung Center for Bio-Tech and Environment Research University of Fisheries 02 Nguyen Dinh Chieu St. Nha Trang City **VIETNAM** Tel: 84 58 83 2065 Fax: 84 58 83 1147 E-mail : huudung@dng.vnn.vn
Lymphocystis	**Prof. Jiang Yulin** Shenzhen Exit and Entry Inspection and Quarantine Bureau 40 Heping Road, Shenzhen 518010 **PEOPLE's REPUBLIC OF CHINA** Tel: 86-755-5592980 Fax:86-755-5588630 E-mail: szapqbxi@public.szptt.net.cn
Diseases of Grass Carp	**Prof. Jiang Yulin** Shenzhen Exit and Entry Inspection and Quarantine Bureau 40 Heping Road, Shenzhen 518010 **PEOPLE's REPUBLIC OF CHINA** Tel: 86-755-5592980 Fax:86-755-5588630 E-mail: szapqbxi@public.szptt.net.cn
Parasitic Diseases	**Dr. Robert D. Adlard** Queensland Museum PO Box 3300, South Brisbane, Queensland 4101 **AUSTRALIA** Tel: +61 7 38407723 Fax: +61 7 38461226 E-mail: RobertAd@qm.qld.gov.au; http://www.qmuseum.qld.gov.au
	Professor R.J.G. Lester Department of Microbiology and Parasitology The University of Queensland, Brisbane 4072 **AUSTRALIA** Tel: +61-7-3365-3305 Fax:+61-7-3365-4620 E-mail:R.Lester@mailbox.uq.edu.au; http://www.biosci.uq.edu.au/micro/academic/lester/lester.htm
	Dr. Ian D. Whittington Department of Microbiology and Parasitology The University of Queensland Brisbane, Queensland 4072 **AUSTRALIA** Tel: 61-7-3365-3302 Fax:61-7-3365-4620 E-mail: i.Whittington@mailbox.uq.edu.au
	Prof. Abu Tweb Abu Ahmed Department of Zoology Dhaka – 100 **BANGLADESH** Tel: 880-2-9666120

	Fax:880-2-8615583 E-mail: zooldu@citechno.net
	Prof. Kazuo Ogawa Department of Aquatic Bioscience Graduate School of Agricultural and Life Sciences The University of Tokyo Yayoi, Bunkyo-ku Tokyo 113-8657 **JAPAN** Tel: 81-3-5841-5282 Fax:81-3-5841-5283 E-mail: aogawak@mail.ec-u-tokyo.ac.jp
	Dr. Bo Young Jee Pathology Division National Fisheries Research and Development Institute 408-1,Shirang-ri, Kitang-up Kitang-gun, Pusan **KOREA RO** Tel: 82-51-720-2498 Fax:82-51-720-2498 E-mail: protjee@nfrdi.re.kr
	Dr. Kim Jeong-Ho Laboratory of Aquatic Animal Diseases College of Veterinary Medicine Chungbuk National University Cheongju, Chungbuk 361-763 **KOREA RO** Tel: +82-43-261-3318 Fax: +82-43-267-3150 E-mail: kimjh89@trut.chungbuk.ac.kr, kimjh89@yahoo.com
	Dr. Craig J. Hayward Laboratory of Aquatic Animal Diseases College of Veterinary Medicine Chungbuk National University Cheongju, Chungbuk 361-763, **KOREA RO** Tel: +82-43-261-2617 Fax: +82-43-267-3150 E-mail: chayward@trut.chungbuk.ac.kr, dameunagi@hotmail.com
	Dr. Susan Lim Lee-Hong Institute of Biological Sciences Institute of Postgraduate Studies and Research Universiti of Malaya 50603 Kuala Lumpur **MALAYSIA** Tel: 603-7594502 Fax: 603-7568940 E-mail: susan@umcsd.um.edu.my
	Prof. Mohammed Shariff Faculty of Veterinary Medicine Universiti Putra Malaysia 43400 Serdang, Selangor **MALAYSIA** Tel: 603-9431064; 9488246 Fax: 603-9488246; 9430626 E-mail: shariff@vet.upm.edu.my

Annex F.AII.List of Regional Resource Experts for Finfish Diseases in Asia Pacific

	Dr. Leong Tak Seng No. 3 Cangkat Minden, Lorong 13 11700 Glugor, Pulau Pinang **MALAYSIA** E-mail: mhpg@pc.jaring.my
	Dr. Tin Tun Department of Zoology University of Mandalay **MYANMAR** E-mail: phys.mdy@mptmail.net.mm
	Dr. Erlinda Lacierda Fish Health Section Aquaculture Department Southeast Asian Fisheries Development Center Tigbauan, Iloilo 5021 **PHILIPPINES** Tel: 63 33 335 1009 Fax: 63 33 335 1008 E-mail: eclacier@aqd.seafdec.org.ph
	Dr. Supranee Chinabut Aquatic Animal Health Research Institute Department of Fisheries Kasetsart University Campus Jatujak, Ladyao, Bangkok 10900 **THAILAND** Tel: 662-5794122 Fax: 662-5613993 E-mail: supranee@fisheries.go.th
	Dr. Melba B. Reantaso Network of Aquaculture Centres in Asia Pacific Department of Fisheries Compound Kasetsart University Campus Jatujak, Ladyao, Bangkok 10900 **THAILAND** Tel: 662- 561-1728 to 9 ext. 113 Fax: 662-561-1727 E-mail: Melba.Reantaso@enaca.org
	Mr. Bui Quang Te Research Institute for Aquaculture No. 1 Dinh Bang, Tu Son, Bac Ninh **VIETNAM**
Bacterial Diseases	**Dr. Indrani Karunasagar** Department of Fishery Microbiology University of Agricultural Sciences Mangalore – 575 002 **INDIA** Tel: 91-824 436384 Fax: 91-824 436384 E-mail: mircen@giasbg01.vsnl.net.in
	Prof. Kiyokuni Muroga Fish Pathology Laboratory Faculty of Applied Biological Science Hiroshima University Higashi-hiroshima 739 **JAPAN** E-mail: fpath@hiroshima-u.ac.jp
	Prof. Mohammed Shariff Faculty of Veterinary Medicine Universiti Putra Malaysia

	43400 Serdang, Selangor **MALAYSIA** Tel: 603-9431064; 9488246 Fax: 603-9488246; 9430626 E-mail: shariff@vet.upm.edu.my
	Mr. Jose O. Paclibare Fish Health Section Bureau of Fisheries and Aquatic Resources Arcadia Building, 860 Quezon Avenue Quezon City, Metro Manila, **PHILIPPINES** Tel/Fax: 632-372-5055 E-mail: jopac@edsamail.com.ph
	Mrs. Celia Lavilla-Torres Fish Health Section Aquaculture Department Southeast Asian Fisheries Development Center Tigbauan, Iloilo 5021 **PHILIPPINES** Tel: 63 33 335 1009 Fax: 63 33 335 1008 E-mail: celiap@aqd.seafdec.org.ph
	Dr. Temdoung Somsiri Aquatic Animal Health Research Institute Department of Fisheries Kasetsart University Campus Jatujak, Ladyao, Bangkok 10900 **THAILAND** Tel: 662-5794122 Fax: 662-5613993 E-mail: temdouns@fisheries.go.th
Viral Diseases	**Mr. Jeong Wan Do** Pathology Division National Fisheries Research and Development Institute 408-1, Shirang-ri, Kitang-up Kitang-gun, Pusan **KOREA RO** Tel: 82-51-720-2481 Fax:82-51-720-2498 E-mail: jwdo@nfrdi.re.kr
	Prof. Jiang Yulin Shenzhen Exit and Entry Inspection and Quarantine Bureau 40 Heping Road, Shenzhen 518010 **PEOPLE's REPUBLIC OF CHINA** Tel: 86-755-5592980 Fax:86-755-5588630 E-mail: szapqbxi@public.szptt.net.cn
	Dr. Gilda Lio-Po Fish Health Section Aquaculture Department Southeast Asian Fisheries Development Center Tigbauan, Iloilo 5021 **PHILIPPINES** Tel: 63 33 335 1009 Fax: 63 33 335 1008 E-mail: liopo@aqd.seafdec.org.ph

Annex F.AII.List of Regional Resource Experts for Finfish Diseases in Asia Pacific

	Dr. Somkiat Kanchanakhan Aquatic Animal Health Research Institute Department of Fisheries Kasetsart University Campus Jatujak, Ladyao, Bangkok 10900 **THAILAND** Tel: 662-5794122 Fax: 662-5613993 E-mail: somkiatkc@fisheries.go.th
Fungal Diseases	**Prof. Kishio Hatai** Divison of Fish Diseases Nippon Veterinary and Animal Science University 1-7-1 Kyonan-cho, Musashino, Tokyo 180 **JAPAN** Tel: 81-0422-31-4151 Fax: 81-0422-33-2094 E-mail: hatai@scan-net.ne.jp
	Dr. Kei Yuasa Fisheries and Aquaculture International Co., Ltd. No. 7 Khoji-machi Bldg., Room B105 4-5 Khoji-machi, Chiyoda-ku Tokyo 102-0083 JAPAN Tel: 81-3-3234-8847 Fax:81-3-3239-8695 E-mail: fai@faiaqua.com; yuasakei@hotmail.com
Finfish Diseases	**Dr. Mark Crane** AAHL Fish Diseases Laboratory Australian Animal Health Laboratory CSIRO Livestock Industries Private Bag 24, Geelong Vic 3220 **AUSTRALIA** Tel : +61 3 52 275118 Fax: +61 3 52 275555 E-mail: mark.crane@li.csiro.au
	Dr. Shuqin Wu Pearl River Fisheries Research Institute Chinese Academy of Fishery Sciences Baihedong, Guangzhou, Guangdong 510380 **PEOPLE's REPUBLIC OF CHINA** Tel: +86 (20) 81517825 ; +86 (20) 81501543 Fax: +86 (20) 81504162 E-mail: sqwxm@163.net
	Dr. Jian-Guo He School of Life Sciences Zhongshan University Guangzhou 510275 **PEOPLE's REPUBLIC OF CHINA** Tel: +86-20-84110976 Fax: +86-20-84036215 Email: lsbrc05@zsu.edu.cn
	Dr. N. Nilakarawasam Department of Zoology The Open University Nawala, Nugegoda **SRI LANKA** Tel.: 094-1-853777 ext. 270 E-mail: nnila@ou.ac.lk

F.AIII. LIST OF USEFUL GUIDES/MANUAL OF FINISH DISEASES IN ASIA-PACIFIC

- **Atlas of Fish Diseases (1989) by Kishio Hatai, Kazuo Ogawa and Hitomi Hirose (eds.) Midori Shobo, Tokyo, 267 p. (in Japanese)**
  Information: Prof. Kazuo Ogawa
  Department of Aquatic Bioscience
  Graduate School of Agricultural and Life Sciences
  The University of Tokyo
  Yayoi, Bunkyo, Tokyo 113-8657
  Tel: +81-3-5841-5282/5284
  Fax: +81-3-5841-5283
  E-mail: aogawak@mail.ecc.u-tokyo.ac.jp

- **Parasites and Diseases of Culture Marine Finfishes in Southeast Asia (1994) by Leong Tak Seng**
  Information: Dr. Leong Tak Seng
  No. 3 Cangkat Minden, Lorong 13
  11700 Glugor, Pulau Pinang
  Malaysia
  E-mail: mhpg@pc.jaring.my

- **Asian Fish Health Bibliography III Japan by Wakabayashi H (editor).** Fish Health Special Publication No. 3. Japanese Society of Fish Pathology, Japan and Fish Health Section of Asian Fisheries Society, Manila, Philippines
  Information: Japanese Society of Fish Pathology

- **Checklist of the Parasites of Fishes of the Philippines by J. Richard Arthur and S. Lumanlan-Mayo.** 1997. FAO Fisheries Technical Paper 369. 102p.
  Information: Dr. Rohana P. Subasinghe
  FAO of the United Nations
  Viale delle Terme di Caracalla
  Rome 00100 Italy
  E-mail: Rohana.Subasinghe@fao.org

- **Manual for Fish Diseases Diagnosis: Marine Fish and Crustacean Diseases in Indonesia (1998) by Zafran, Des Roza, Isti Koesharyani, Fris Johnny and Kei Yuasa**
  Information: Gondol Research Station for Coastal Fisheries
  P.O. Box 140 Singaraja, Bali, Indonesia
  Tel: (62) 362 92278
  Fax: (62) 362 92272

- **Diagnostic Procedures for Finfish Diseases (1999) by Kamonporn Tonguthai, Supranee Chinabut, Temdoung Somsiri, Pornlerd Chanratchakool and Somkiat Kanchanakhan**
  Information: Aquatic Animal Health Research Institute
  Department of Fisheries
  Kasetsart University Campus
  Jatujak, Ladyao, Bangkok 10900
  THAILAND
  Tel: (66.2) 579.41.22
  Fax: (66.2) 561.39.93
  E-mail: aahri@fisheries.go.th

- **Pathology and Histopathology of Epizootic Ulcerative Syndrome (EUS) by Supranee Chinabut and RJ Roberts**
  Information: Aquatic Animal Health Research Institute
  Department of Fisheries
  Kasetsart University Campus
  Jatujak, Ladyao, Bangkok 10900
  THAILAND
  Tel: (66.2) 579.41.22
  Fax: (66.2) 561.39.93

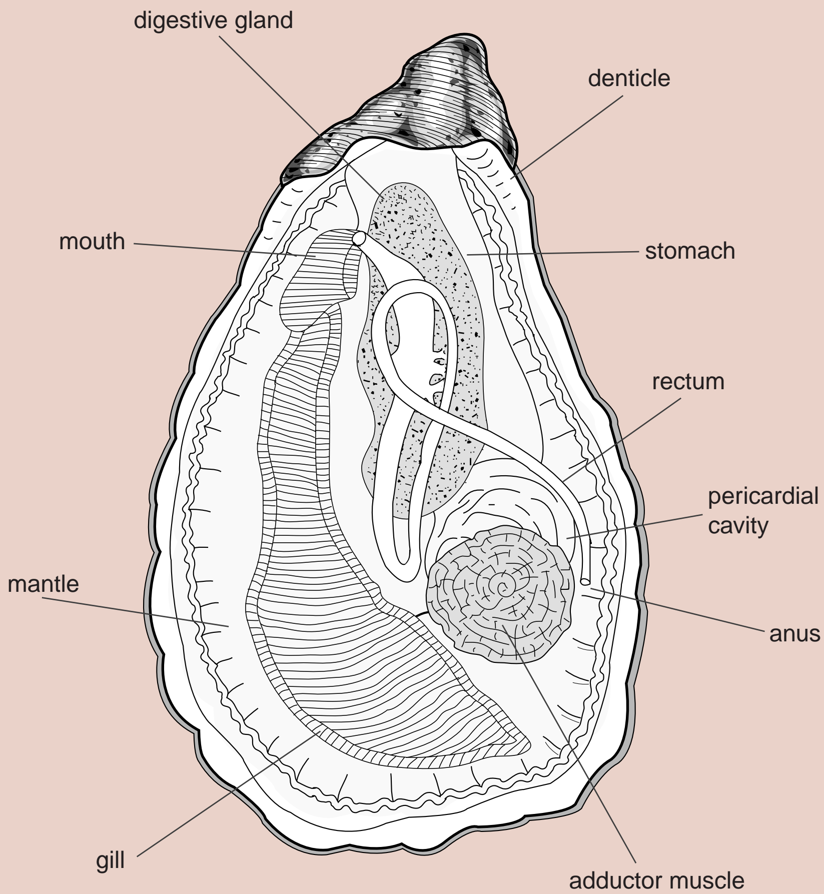
E-mail: aahri@fisheries.go.th

- **Fish Health for Fisfarmers (1999) by Tina Thorne**
  Information: Fisheries Western Australia
  3rd Floor, SGIO Atrium
  186 St. Georges Terrace, Perth WA 6000
  Tel: (08) 9482 7333
  Fax: (08) 9482 7389
  Web: http://www.gov.au.westfish

- **Australian Aquatic Animal Disease – Identification Field Guide (1999) by Alistair Herfort and Grant Rawlin**
  Information: AFFA Shopfront – Agriculture, Fisheries and Forestry – Australia
  GPO Box 858, Canberra, ACT 2601
  Tel: (02) 6272 5550 or free call: 1800 020 157
  Fax: (02) 6272 5771
  E-mail: shopfront@affa.gov.au

- **Manual for Fish Disease Diagnosis - II: Marine Fish and Crustacean Diseases in Indone sia (2001) by Isti Koesharyani, Des Roza, Ketut Mahardika, Fris Johnny, Zafran and Kei Yuasa, edited by K. Sugama, K. Hatai, and T Nakai**
  Information: Gondol Research Station for Coastal Fisheries
  P.O. Box 140 Singaraja, Bali, Indonesia
  Tel: (62) 362 92278
  Fax: (62) 362 92272

Basic Anatomy of an Oyster



Basic anatomy of an oyster.

SECTION 3 - MOLLUSCAN DISEASES

Basic Anatomy of an Oyster		108
SECTION 3 - MOLLUSCAN DISEASES		
M.1	**GENERAL TECHNIQUES**	110
M.1.1	**Gross Observations**	111
M.1.1.1	*Behaviour*	111
M.1.1.2	*Shell Surface Observations*	111
M.1.1.3	*Inner Shell Observations*	111
M.1.1.4	*Soft-Tissue Surfaces*	114
M.1.2	**Environmental Parameters**	114
M.1.3	**General Procedures**	116
M.1.3.1	*Pre-Collection Preparation*	116
M.1.3.2	*Background Information*	116
M.1.3.3	*Sample Collection for Health Surveillance*	116
M.1.3.4	*Sample Collection for Disease Diagnosis*	116
M.1.3.5	*Live Specimen Collection for Shipping*	116
M.1.3.6	*Preservation of Tissue Samples*	117
M.1.3.7	*Shipping Preserved Samples*	118
M.1.4	**Record Keeping**	118
M.1.4.1	*Gross Observations*	118
M.1.4.2	*Environmental Observations*	119
M.1.4.3	*Stocking Records*	119
M.1.5	**References**	119
	DISEASES OF MOLLUSCS	
M.2	**Bonamiosis (*Bonamia sp., B. ostreae*)**	121
M.3	**Marteiliosis (*Marteilia refringens, M. sydneyi*)**	125
M.4	**Mikrocytosis (*Mikrocytos mackini, M. roughleyi*)**	129
M.5	**Perkinsosis (*Perkinsus marinus, P. olseni*)**	133
M.6	**Haplosporidiosis (*Haplosporidium costale, H. nelsoni*)**	138
M.7	**Marteilioidosis (*Marteilioides chungmuensis, M. branchialis*)**	144
M.8	**Iridovirosis (*Oyster Velar Virus Disease*)**	147
	ANNEXES	
M.AI	**OIE Reference Laboratories for Molluscan Diseases**	149
M.AII	**List of Regional Resource Experts for Molluscan Diseases in Asia-Pacific**	150
M.AIII	**List of Useful Diagnostic Guides/Manuals to Molluscan Health**	152

M.1 GENERAL TECHNIQUES

(SE McGladdery)

Fig.M.1.1.1. Gaping hard shell clam, *Mercenaria mercenaria*, despite air exposure and mechnical tapping.

(MG Bondad-Reantaso)

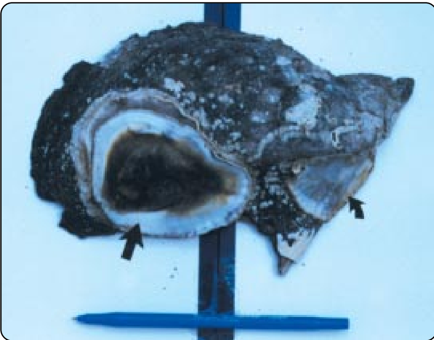


Fig.M.1.1.2a. Mollusc encrustment (arrows) of winged oyster, Pteria penguin, Guian Pearl Farm, Eastern Samar, Philippines (1996).

(D Ladra)

Fig.M.1.1.2b. *Pteria penguin* cultured at Guian Pearl Farm, Eastern Samar, Philippines with extensive shell damage due to clionid (boring) sponge (1992).

Fig.M.1.1.2f. Winged oyster, *Pteria penguin*, shell with clionid sponge damage. Guian Pearl Farm, Eastern, Philippines (1996).

(MG Bondad- Reantaso)

Fig.M.1.1.2c,d. *Pteria penguin* shell with dense multi-taxa fouling, Guian Pearl Farm, Eastern Philippines (1996).

(SE McGladdery)

Fig.M.1.1.2e. *Polydora* sp. tunnels and shell damage at hinge of American oyster, *Crassostrea virginica*, plus barnacle encrusting of other shell surfaces.

(MG Bondad-Reantaso)

M.1 General Techniques

General molluscan health advice and other valuable information are available from the OIE Reference Laboratories, Regional Resource Experts in the Asia-Pacific, FAO and NACA. A list is provided in Annexes M.A1 and M.AII, and up-to-date contact information may be obtained from the NACA Secretariat in Bangkok (e-mail: naca@enaca.org). Other useful guides to diagnostic procedures which provide valuable references for molluscan diseases are listed in Annex M.AIII.

M.1.1 **Gross Observations**

M.1.1.1 *Behaviour* (Level I)

It is difficult to observe behavioural changes in molluscs in open-water, however, close attention can be made of behaviour of both broodstock and larvae in hatcheries. Since disease situations can erupt very quickly under hatchery conditions, regular and close monitoring is worth Level I efforts (see Iridovirus - M.8).

Feeding behaviour of larval molluscs is also a good indicator of general health. Food accumulation in larval tanks should be noted and samples of larvae examined, live, under a dissecting microscope for saprobiotic fungi and protists (*e.g.* ciliates) and/or bacterial swarms. Pre-settlement stages may settle to the bottom prematurely or show passive circulation with the water flow currents in the holding tanks.

Juvenile and adult molluscs may also cease feeding, and this should be cause for concern under normal holding conditions. If feeding does not resume and molluscs show signs of weakening (days to weeks depending on water temperature) samples should be collected for laboratory examination. Signs of weakening include gaping (*i.e.* bivalve shells do not close when the mollusc is touched or removed from the water) (Fig. M.1.1.1), accumulation of sand and debris in the mantle and on the gills, mantle retraction away from the edge of the shell, and decreased movement in mobile species (*e.g.* scallop swimming, clam burrowing, abalone grazing, *etc.*).

Open-water mortalities that assume levels of concern to the grower should be monitored to determine if there are any patterns to the losses. Sporadic moralities following periods of intense handling should be monitored with minimal additional handling if at all possible. If the mortalities persist, or increase, samples should be collected for laboratory analysis. Mortalities that appear to have a uniform distribution should be examined immediately and environmental factors pre- and post-mortality recorded. Mortalities that appear to spread from one area to another suggest the presence of an infectious disease agent and should be sampled immediately. Affected animals should be kept as far away as possible from unaffected animals until the cause of the mortalities can be determined.

M.1.1.2 *Shell Surface Observations* (Level I)

Fouling organisms (barnacles, limpets, sponges, polychaete worms, bivalve larvae, tunicates, bryozoans, *etc.*) are common colonists of mollusc shell surfaces and do not normally present a threat to the health of the mollusc (Fig. M.1.1.2a,b). Suspension and shallow water culture, however, can increase exposure to fouling and shells may become covered by other animals and plants (Fig. M.1.1.2c,d). This can affect health directly by impeding shell opening and closing (smothering) or indirectly through competition for food resources. Both circumstances can weaken the mollusc so cleaning may be required. Such defouling should be undertaken as rapidly as possible, to minimise the period of removal from the water, during cooler periods of the day. Rapid cleaning is usually achieved using high pressure water or mechanical scapers. Defouled molluscs should be returned to clean water. Fouling organisms should not be discarded in the same area as the molluscs, since this will accelerate recolonisation. Signs of weakening that persist or increase after cleaning, should be investigated further by laboratory examination.

Shell damage by boring organisms, such as sponges and polychaete worms (Fig. M.1.1.2e, f) is normal in open-water growing conditions. Although usually benign, under certain conditions (especially in older molluscs) shells may be rendered brittle or even become perforated. Such damage can weaken the mollusc and render it susceptible to pathogen infections.

Shell deformities (shape, holes in the surface), fragility, breakage or repair should be noted, but are not usually indicative of a disease condition (Fig.M.1.1.2g, h). Abnormal colouration and smell, however, may indicate a possible soft-tissue infection which may require laboratory examination.

M.1.1.3 *Inner Shell Observations* (Level I)

The presence of fouling organisms (barnacles, sponges, polychaete worms, *etc.*) on the inner shell surface is a clear indication of a weak/

(MG Bondad-Reantaso)

Fig.M.1.1.2g,h. *Pinctada maxima*, shell with clionid sponge damage due to excavation of tunnels exhalent-inhalent openings (holes) to the surface (arrows). Other holes (small arrows) are also present that may have been caused by polychaetes, gastropod molluscs or other fouling organisms. Guian Pearl Farm, Eastern Philippines (1996).

(MG Bondad-Reantaso)

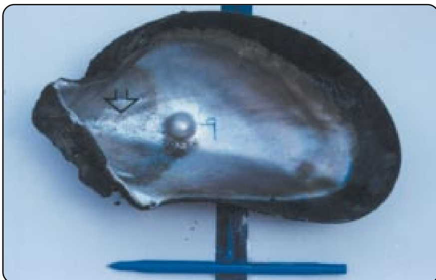


Fig.M.1.1.3a. Winged oyster, *Pteria penguin*, shell showing clionid sponge damage through to the inner shell surfaces, Guian Pearl Farm, Eastern Philippines (1996).

(B Jones)

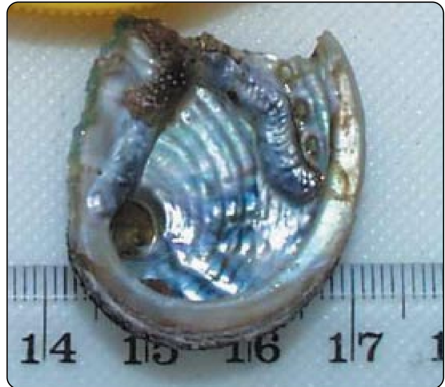


Fig.M.1.1.3a1. Abalone (*Haliotis roei*) from a batch killed by polydoriid worms.

(D Ladra)

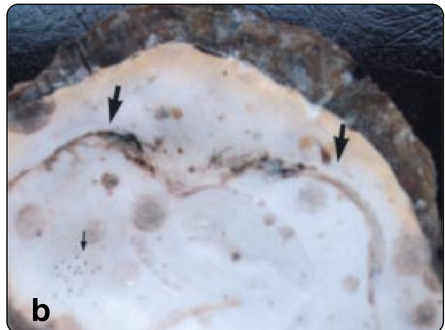
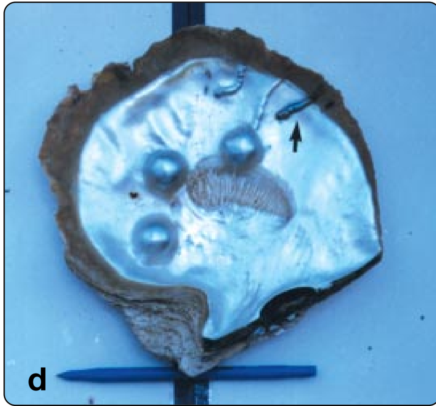


Fig.M.1.1.3b,c. b. Shells of *Pinctada maxima* showing a erosion of the nacreous inner surfaces (arrows), probably related to chronic mantle retraction; c. Inner surface of shell showing complete penetration by boring sponges (thin arrows).

(MG Bondad-Reantaso)



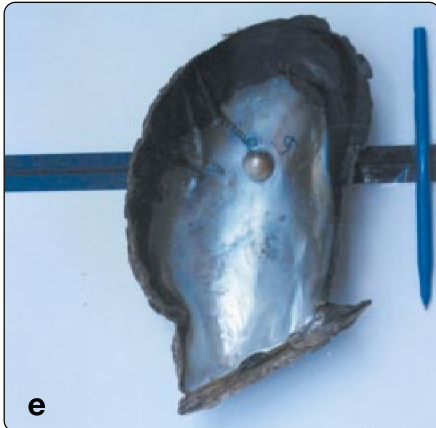


Fig.M.1.1.3d,e,f. *Pinctada maxima* (d), *Pteria penguin* (e) and edible oyster (*Crassostrea* sp.) (f) shells showing *Polydora*-related tunnel damage that has led to the formation of mud-filled blisters.

(MG Bondad-Reantaso)

Fig.M.1.1.3g. Inner shell of winged pearl oyster showing: tunnels at edge of the shell (straight thick arrow); light sponge tunnel excavation (transparent arrow); and blisters (small thick arrow) at the adductor muscle attachment site. Guian Pearl Farm, Eastern Philippines (1996).

(SE McGladdery)

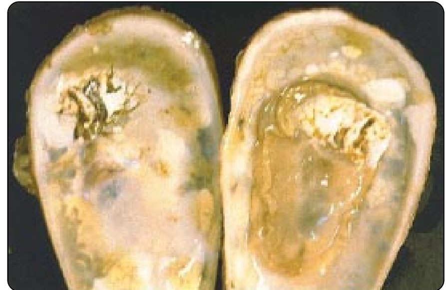


Fig.M.1.1.3.h. Extensive shell penetration by polychaetes and sponges causing weakening and retraction of soft-tissues away from the shell margin of an American oyster *Crassostrea virginica.*

sick mollusc (Fig.M.1.1.3a and Fig.M.1.1.3a1). The inner surfaces are usually kept clean through mantle and gill action. Perforation of the inner surface can be sealed off by deposition of additional conchiolin and nacre (Fig.M.1.1.3b,c). This may result in the formation of a mud- or water-filled "blister" (Fig.M.1.1.3d,e,f). Shell coverage can also occur over irritants attached to, or lying up against the inner shell, a process that may result in a "blister pearl" (Fig.M.1.1.3g).

Where perforation or other irritants exceed repair, the health of the mollusc is jeopardised and it becomes susceptible to opportunistic infections (Fig.M.1.1.3.h). The degree of shell perforation can be determined by holding the shell up to a strong light.

Where abnormalities occurring within the matrix of the shell warrant further investigation, freshly collected specimens can be sent intact to the laboratory or fixed for subsequent decalcification, as required.

M.1.1.4 *Soft-Tissue Surfaces* (Level I)

The appearance of the soft-tissues is frequently indicative of the physiological condition of the animal. Gross features which should be recorded include:

- condition of the animal as listed below:

  fat - the soft-tissues fill the shell, are turgid and opaque
  medium - the tissues are more flaccid, opaque and may not fill the shell cavity
  watery - the soft-tissues are watery/transparent and may not fill the shell cavity
  (Fig.M.1.1.4a, Fig.M.1.1.4b)

- colour of the digestive gland – *e.g.*, pale, mottled, dark olive
- any abnormal enlargement of the heart or pericardial cavity – *e.g.,* cardiac vibriosis, tumours
- presence of focal lesions such as:

  abnormal coloration (*eg.*, patches of green, pink, red, black, etc.)
  abscesses (Fig. M.1.1.4c)
  tumour-like lesions (Fig. M.1.1.4d)
  tissue (*e.g.* gill) erosion
- presence of water blisters in the viscera, palp, or mantle (Fig.M.1.1.4e)
- presence of pearls or other calcareous deposits (Fig.M.1.1.4f) within the soft tissues
- presence of parasites or commensals such as:

  pea crabs in mantle cavity
  parasitic copepods attached to gills
  polychaetes, nematodes and turbellarians in mantle cavity or on surrounding surfaces (Fig. M.1.1.4g)
  redworm (*Mytilicola* spp.) usually exposed only on dissection of the digestive tract
  ciliates (sessile or free-swimming) and other protistans (for larvae only)
  bacteria (for larvae only)

- *any* mechanical (*e.g.,* knife) damage to the soft-tissues during the opening of the shell.

Abscess lesions, pustules, tissue discolouration, pearls, oedema (water blisters), overall transparency or wateriness, gill deformities, *etc.*, can be present in healthy molluscs, but, if associated with weak or dying animals, should be cause for concern. Record the levels of tissue damage and collect samples of both affected and unaffected animals for laboratory examination. Moribund animals, or those with foul-smelling tissues may be of little use for subsequent examination (especially from warm water conditions), however, numbers affected should be recorded.

Worms or other organisms (*e.g.* pea crabs, copepods, turbellarians) on the soft-tissues are also common and not generally associated with disease. If present in high numbers on weak molluscs, however, numbers should be noted and samples of intact specimens collected for laboratory examination and identification. Fixation in 10% buffered formalin is usually adequate for preserving the features necessary for subsequent identification.

M.1.2 **Environmental Parameters (Level I)**

Environmental conditions have a significant effect on molluscan health, both directly (within the ranges of physiological tolerances) and indirectly (enhancing susceptibility to infections). This is especially important for species grown under conditions which differ significantly from the wild (*e.g.* oysters grown in suspension). Important environmental factors for molluscan health include water temperature, salinity, turbidity, fouling and plankton blooms. Extremes and/or rapid fluctuations in these can seriously compromise molluscan health. Anthropogenic factors include a wide range of biologic and chemical pollutants. Since molluscs are, essentially, sessile species (especially under culture conditions) this renders them particularly susceptible to pollution. In addition, molluscs have

(SE McGladdery)

Fig.M.1.1.4a. Normal oyster (*Crassostrea virginica*) tissues.

(SE McGladdery)

Fig.M.1.1.4b. Watery oyster (*Crassostrea virginica*) tissues – compare with M.1.1.4a.

(SE McGladdery)

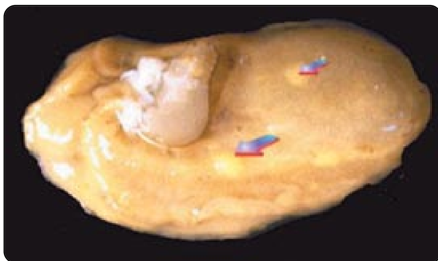
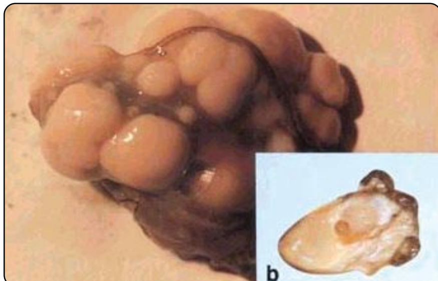


Fig.M.1.1.4c. Abscess lesions (creamy-yellow spots, see arrows) in the mantle tissue of a Pacific oyster (*Crassostrea gigas*).

(MS Park and DL Choi)



(SE McGladdery)

Fig.M.1.1.4e. Water blister (oedema/edema) in the soft-tissues of the mantle margin of an American oyster (*Crassostrea virginica*).

(SE McGladdery)

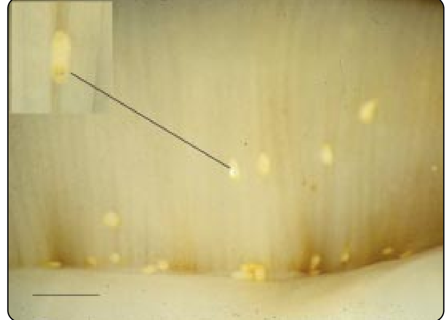


Fig.M.1.1.4f. Calcareous deposits (“pearls”) in the mantle tissues of mussels in response to irritants such as mud or digenean flatworm cysts.

(SE McGladdery and M Stephenson)

Fig.M.1.1.4g. Polydoriid tunnels underlying the nacre at the inner edge of an American oyster (*Crassostrea virginica*) shell, plus another free-living polychaete, *Nereis diversicolor* on the inner shell surface.

<

Fig.M.1.1.4d. Gross surface lesions in Pacific oyster (*Crassostrea gigas*) due to *Marteiliodes chungmuensis.*

low tolerance of some other water-uses/abuses (*e.g.*, dynamite and cyanide fishing; dragging; creosote and other anti-fouling chemical compounds; agricultural run-off).

Maintaining records of temperature, salinity (in estuarine or coastal areas), turbidity and man-made disturbances provide valuable background data, essential for accurate interpretation of mortality observations and results from laboratory analyses.

M.1.3 **General Procedures**

M.1.3.1 *Pre-Collection Preparation*

Wherever possible, check the number of specimens required for laboratory examination with laboratory personnel *before* collecting the sample. Ensure that each specimen is intact, *i.e.*, no empty or mud-filled shells. Larger sample numbers are generally needed for screening purposes compared with numbers required for disease diagnosis.

M.1.3.2 *Background Information (Level I)*

All samples being submitted for laboratory examination should include as much background information as possible, such as:

- reason(s) for submitting the sample (mortali ties, abnormal growth/spawning, health screening, *etc.*);
- gross observations and environmental para eters (as described under M.1.1 and M.1.2);
- where samples are submitted due to mortal ties, approximate prevalences and patterns of mortality (acute or chronic/sporadic cumulative losses), and
- whether or not the molluscs are from local populations or from another site. If the stock is not local, the source and date of transfer should also be noted.

The above information will help identify if handling stress, change of environment or infectious agents may be a factor in mortalities. It will also help speed up accurate diagnosis of a disease problem or disease-risk analysis.

M.1.3.3 *Sample Collection for Health Surveillance*

The most important factors associated with collection of specimens for surveillance are:

- sample numbers that are high enough (see Table M.1.3.3 below)
- susceptible species are sampled
- samples include age- or size-groups that are most likely to manifest detetctable infections. Such information is given under specific disease sections.

The standard sample sizes for screening healthy aquatic animals, including molluscs, is given in Table M.1.3.3 below.

M.1.3.4 *Sample Collection for Disease Diagnosis*

All samples submitted for disease diagnosis should include as much supporting information as possible including:

- reason(s) for submitting the sample (mortalities, abnormal growth, *etc.*)
- handling activities (de-fouling, size sorting/ grading, site changes, new species/stock introduction, *etc.*)
- history and origin(s) of the affected population(s);
- environmental changes

M.1.3.5 *Live Specimen Collection for Shipping* (Level I)

Once the required number of specimens has been determined, and the laboratory has provided a date or time for receipt of the sample, the molluscs should be collected from the water. This should take place as close to shipping as possible to reduce air-storage changes in tissues and possible mortalities during transportation. This is especially important for moribund or diseased mollusc samples.

The laboratory should be informed of the estimated time of arrival to ensure they have the materials required to process the sample prepared before the sample arrives. This helps reduce the time between removal from the water and preservation of the specimens for examination.

The molluscs should be wrapped in paper soaked with ambient seawater. For small seed (<10 mm), these can be packed in paper or styrofoam cups along with damp paper towel to prevent movement during transportation. Larger molluscs should be shipped in insulated and sealable (leakproof) coolers (styrofoam or plastic). Where more than one sample is included in the same cooler, each should be placed in a separate and *clearly* labeled plastic bag (tied or ziploc). Use of plastic bags is required to prevent exposure of marine species

Population Size	Prevalence (%) 0.5	1.0	**2.0**	3.0	4.0	**5.0**	10.0
50	46	46	46	37	37	29	20
100	93	93	76	61	50	43	23
250	192	156	110	75	62	49	25
500	314	223	127	88	67	54	26
1000	448	256	136	92	69	55	27
2500	512	279	142	95	71	56	27
5000	562	288	145	96	71	57	27
10000	579	292	146	96	72	29	27
100000	594	296	147	97	72	57	27
1000000	596	297	147	97	72	57	27
>1000000	600	300	**150**	100	75	**60**	30

Table M.1.3.3[1]. Sample sizes needed to detect at least one infected host in a population of a given size, at a given prevalence of infection. Assumptions of 2% and 5% prevalences are most commonly used for surveillance of presumed exotic pathogens, with a 95% confidence limit.

to freshwater ice (gel-paks or plastic bottles containing frozen water are recommended over loose ice to keep specimens cool) and to reduce loss of mantle fluids.

Label containers clearly:

"Live Specimens, Store at _____°C to ______°C DO NOT FREEZE"

If being shipped by air also indicate:

"HOLD AT AIRPORT AND CALL FOR PICK-UP"

Clearly indicate the name and telephone number of the contact person responsible for picking up the package at the airport or receiving it at the laboratory.
Ship ***early in the week*** to avoid arrival during the weekend with possible loss of samples due to improper storage. Inform the contact person as soon as the shipment has been sent and, where appropriate, give them the name of the carrier and waybill number.

M.1.3.6 *Preservation (Fixation) of Tissue Samples* (Level I - with basic training)

For samples that cannot be delivered live to a diagnostic laboratory, due to distance or slow transportation, specimens should be fixed (preserved) on site. This is suitable for subsequent histology examination, but means that routine bacteriology, mycology or media culture (*e.g.,* Fluid Thioglycollate Medium culture of *Perkinsus* spp.) cannot be performed. Diagnostic needs should, therefore, be discussed with laboratory personnel prior to collecting the sample.

The following fixatives can be used for preservation of samples:

i) 1G4F solution (1% Glutaraldehyde : 4% Formaldehyde)

*Stock 1G4F solution - may be held at 4oC for up to 3 months:

120 ml	37-40% buffered formalin solution**
20 ml	50% glutaraldehyde
360 ml	tap water

**Buffered formalin solution:

1 litre	37-40% formaldehyde
15 gm	disodium phosphate (Na_2HPO_4)

[1] Ossiander, F.J. and G. Wedermeyer. 1973. Journal of Fisheries Research Board of Canada 30:1383-1384.

0.06 gm	sodium hydroxide (NaOH)
0.03 gm	phenol red (pH indicator)

Working solution – should be prepared immediately prior to use:

500 ml	filtered ambient seawater or Instant Ocean
500 ml	Stock 1G4F solution*

The required tissue thickness is about 2-3 mm. Tissues can tolerate long storage in this fixative at room temperature. (N.B. Thicker tissues, or whole animals, may be fixed using the 10% buffered formalin solution as described below).

ii) 10% Buffered formalin in filtered ambient seawater (This is the easiest solution to prepare and store).

10 ml	37-40% buffered formalin solution**
90 ml	filtered ambient seawater

N.B. Whole specimens less than 10 mm thick can be fixed with this solution. If the specimens are larger, cut them into two or more pieces before fixing (ensure that pieces from different specimens do not get mixed up).

iii) Davidson's Fixative

Tissue up to 10 mm in thickness can be fixed in Davidson's fixative. Prior to embedding, tissues need to be transferred to either 50% ethanol for 2 hours (minimum) and then to 70% ethanol, or directly to 70% isopropanol. Best results are obtained if fixative is made up in the following order of ingredients.

Stock Solution:

400 ml	glycerin
800 ml	formalin(37-40% formaldhyde)
1200 ml	95% ethanol (or 99% iso propanol)
1200 ml	filtered natural or artificial seawater

Working Solution: dilute 9 parts stock solution with 1 part glacial acetic acid

Important Notes:

- All fixatives should be kept away from open water and used with caution against contact with skin and eyes.
- If the molluscs cannot be fixed intact, contact the diagnostic laboratory to get guidance for cracking shell hinges or removing the required tissues.

M.1.3.7 *Shipping Preserved Samples* (Level I)

Many transport companies (especially air carriers) have strict regulations regarding shipping any chemicals, including fixed samples for diagnostic examination. Check with the carrier *before* collecting the sample to prevent loss of time and/or specimens due to inappropriate packaging, labeling, *etc.*. If the tissues have been adequately fixed (as described in M.1.3.4), most fixative or storage solution can be drained from the sample for shipping purposes. As long as sufficient solution is left to keep the tissues from drying out, this will minimise the quantity of chemical solution being shipped. Pack fixed samples in a durable, leak-proof container.

Label containers clearly with the information as described for live specimens (M.1.3.3.). Clearly indicate the name and telephone number of the contact person responsible for picking up the package at the airport or receiving it at the laboratory. Ship **early in the week** to avoid arrival during the weekend with possible loss of samples due to improper storage. Inform the contact person as soon as the shipment has been sent and, where appropriate, give them the name of the carrier and waybill number.

If being shipped by air also indicate:

"HOLD AT AIRPORT AND CALL FOR PICK-UP"

M.1.4 **Record-Keeping** (Level I)

Record keeping is essential for effective disease management. For molluscs, many of the factors that should be recorded are outlined in sections M.1.4.1, M.1.4.2, and M.1.4.3.

M.1.4.1 *Gross Observations* (Level I)

Gross observations can be included with routine monitoring of mollusc growth, either by sub-sampling from suspension cages, lines or stakes, or by guess estimates from surface observations.

For hatchery operations, the minimum essential information which should be recorded/logged are:

- feeding activity
- growth
- mortalities

These observations should be recorded on a daily basis for larval and juvenile molluscs, including date, time, tank, broodstock (where

there are more than one) and food-source (algal culture batch or other food-source). Dates and times for tank and water changes should also be noted, as well as dates and times for pipe flushing and/or disinfection. Ideally, these logs should be checked regularly by the person responsible for the site/animals.

For open-water mollusc sites, the minimum essential observations which need to be recorded/ logged include:

- growth
- fouling
- mortalities

These should be recorded with date, site location and any action if taken (*e.g.*, defouling or sample collection for laboratory examination). Ideally, these logs should be checked regularly by the person responsible for the site/animals.

M.1.4.2 *Environmental Observations* (Level I)

This is most applicable to open water sites, but should also be included in land-based systems with flow-through or well-based water sources. The minimum essential data which should be recorded are:

- temperature
- salinity
- turbidity (qualitative evaluation or secchi disc)
- algal blooms
- human activity

The frequency of these observations will vary with site. Where salinity or turbidity rarely vary, records may only be required during rainy seasons or exceptional weather conditions. Temperate climates will require more frequent water temperature monitoring than tropical climates. Human activity should be logged on an "as it happens" basis for reference if no infections or natural environmental changes can be attributed to a disease situation.

M.1.4.3 *Stocking Records* (Level I)

Information on movements of molluscs into and out of a hatchery should be recorded. This should include:

- exact source of the broodstock/seed
- condition on arrival
- date, time and person responsible for receiving delivery of the stock
- date, time and destination of stock shipped out of the hatchery

Where possible, animals from different sources should not be mixed.

All movements of molluscs onto and off an open-water site should also be recorded, including:

- exact source of the molluscs
- condition on arrival
- date, time and person responsible for receiving delivery of the stock
- date, time and destination of stock shipped off site

In addition, all movements of stocks within a hatchery, nursery or grow out site should be logged with the date for tracking purposes if a disease situation arises.

M.1.5 **References**

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MOLLUSCAN DISEASES
M.2 BONAMIOSIS
(*BONAMIA* SP., *B. OSTREAE*)

M.2.1 **Background Information**

M.2.1.1 *Causative Agents*

Bonamiosis (*a.k.a.* Microcell Disease; haemocyte disease of flat or dredge oysters) is caused by two Protistan (= Protozoan = single-celled) species belonging to the Haplosporidia: *Bonamia ostreae* and *Bonamia* sp.. More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.2.1.2 *Host Range*

Bonamia ostreae occurs naturally in *Ostrea edulis* (European oyster) and *O. conchaphila* (*O. lurida*) (Olympia oyster). Other ostreiid species can become infected if transferred to enzootic areas, namely *O. puelchana*, *O. angasi* and *Ostrea lutaria* (*Tiostrea lutaria*) (New Zealand oyster), *Tiostrea chilensis* (*Ostrea chilensis*) (South American oyster), thus, all species of *Ostrea, Tiostrea* and some *Crassostrea* (*C. ariakensis*) should be considered susceptible. To date, *Crassostrea gigas* (Pacific oyster), *Mytilus edulis* and *M. galloprovincialis* (edible mussels) and *Ruditapes decussatus* and *R. philippinarum* (European and Manila clams) have been found to be resistant to infection. These species have also been shown to be incapable of acting as reservoirs or sub-clinical carriers of infection.

M.2.1.3 *Geographic Distribution*

Bonamia ostreae: The Netherlands, France, Spain, Italy, Ireland, the United Kingdom (excluding Scotland) and the United States of America (States of California, Maine and Washington). Denmark, although stocked with infected oysters in the early 1980's, has shown no sign of persistence of the infection and their European oysters are now considered to be free of *B. ostreae*.

Bonamia sp.: Australia (Western Australia, Victoria and Tasmania) and New Zealand (South Island and southern North Island).

M.2.1.3 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999 to 2000)*

For the reporting year 1999, *Bonamia* sp. was positively reported in Australia in April, July and October in Tasmania; July and October in Western Australia. For the year 2000, *Bonamia* sp. was reported in March and April in Western Australia. In New Zealand, *Bonamia* sp. was reported every month for 1999 and 2000 reporting periods (OIE 1999, OIE 2000b).

M.2.2 **Clinical Aspects**

Most infections show no clinical signs until the parasites have proliferated to a level that elicits massive blood cell (haemocyte) infiltration and diapedesis (Fig.M.2.2a). The pathology of infection varies with the species of *Bonamia,* and with host species. *Bonamia ostreae* infects the haemocytes of European oysters (Fig.M.2.2b), where it divides until the haemocyte bursts, releasing the parasites into the haemolymph. Infections likely occur through the digestive tract, but gill infections suggest this may also be another infection route and macroscopic gill lesions are sometimes visible. The pathology of *Bonamia* sp. in Australian *Ostrea angasi* and New Zealand populations of *Tiostrea chilensis* is very different. In Australia's *O. angasi*, the first indication of infection is high mortality. Surviving oysters rapidly start to gape on removal from the water and may have "watery" tissues and a ragged appearance to the margin of the gill (unpublished data, B. Jones, Fisheries Western Australia). *Bonamia* sp. infects the walls of the gills, digestive ducts and tubules (Fig.M.2.2c), from which the parasites may be released into the gut or surrounding water. Infected haemocytes may contain up to 6 *Bonamia* parasites (Fig.M.2.2d). Infections induce massive abscess-like lesions (haemocytosis), even in the presence of only a few parasites. In *T. chilensis, Bonamia* sp. appears to enter via the gut wall (Fig.M.2.2e), and then infects the haemocytes, where up to 18 *Bonamia* per haemocyte can be found (Fig.M.2.2f). The resultant haemocytosis is less severe than in *O. angasi.* When infected haemocytes enter the gonad of *T. chilensis* to reabsorb unspawned gametes, the parasites proliferate and may be released via the gonoduct. Alternative release is also possible via tissue necrosis following the death of the host. Despite the differences in pathology, gene sequencing studies (unpublished data, R. Adlard, University of Queensland, Australia) have shown that the Australian and New Zealand *Bonamia* sp. are the same species.

M.2.3 **Screening Methods**

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.2 Bonamiosis (*Bonamia* sp., *B. ostreae*)

(SE McGladdery)

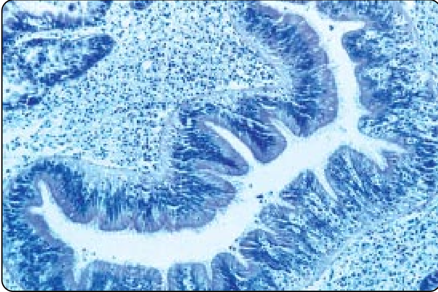


Fig.M.2.2a. Haemocyte infiltration and diapedesis across intestinal wall of a European oyster (*Ostrea edulis*) infected by *Bonamia ostreae.*

(SE McGladdery)

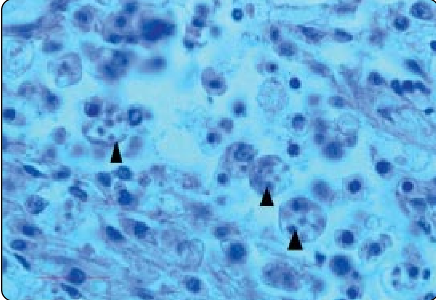


Fig.M.2.2b. Oil immersion of *Bonamia ostreae* inside European oyster (*Ostrea edulis*) haemocytes (arrows). Scale bar 20 µm.

(PM Hine)

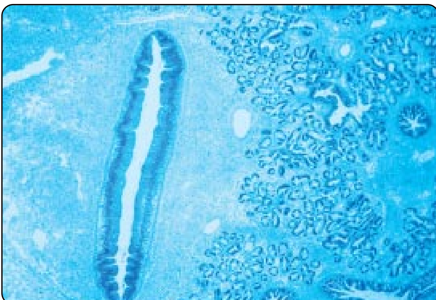


Fig.M.2.2c. Systemic blood cell infiltration in Australian flat oyster (*Ostrea angasi*) infected by *Bonamia* sp. Note vacuolised appearance of base of intestinal loop and duct walls (H&E).

Fig.M.2.2f. Oil immersion of haemocytes packed with *Bonamia* sp. (arrows) in an infected *Tiostrea lutaria* (H&E).

(PM Hine)

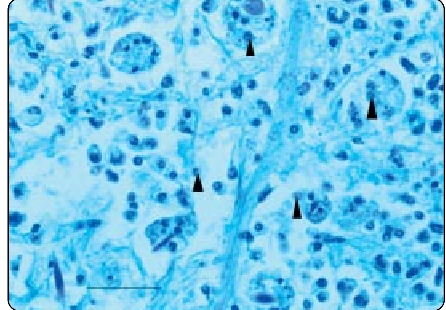


Fig.M.2.2d. Oil immersion of *Bonamia* sp. infecting blood cells and lying free (arrows) in the haemolymph of an infected Australian flat oyster, *Ostrea angasi*. Scale bar 20µm (H&E).

(PM Hine)

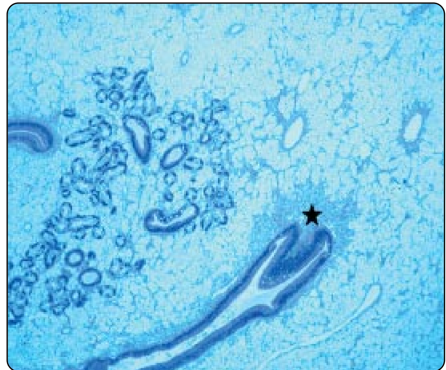
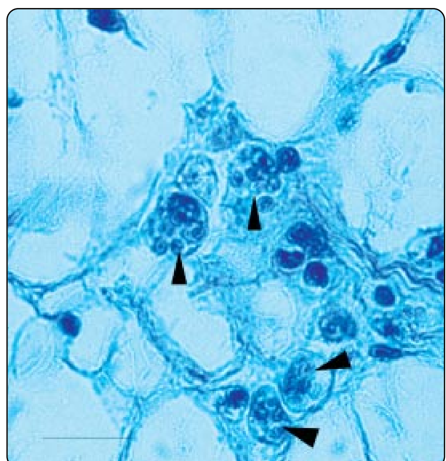


Fig.M.2.2e. Focal infiltration of haemocytes around gut wall (star) of *Tiostrea lutaria* (New Zealand flat oyster) typical of infection by *Bonamia* sp. (H&E).

(PM Hine)



M.2.3.1 *Presumptive*

M.2.3.1.1 Gross Observations (Level I).

Slowed growth, presence of gill lesions (in some cases), gaping and mortalities of *Ostrea edulis* should be considered suspect for Bonamiosis. Gross signs are not disease specific and require Level II examination.

M.2.3.1.2 Cytological Examination (Level II)

Spat or heart (preferably ventricle) smears or impressions (dabs) can be made onto a clean microscope slide and air-dried. Once dry, the preparation is fixed in 70% methanol. Quick and effective staining can be achieved using commercially available blood-staining (cytological) kits, following the manufacturer's instructions. The stained slides are then rinsed (gently) in tapwater, allowed to dry and cover-slipped using a synthetic resin mounting medium. The parasite has basophilic (or colourless - *Bonamia* sp. in *O. angasi*) cytoplasm and an eosinophilic nucleus (depending on the stain used). An oil immersion observation time of 10 mins per oyster preparation is considered sufficient for screening cytology, tissue imprint and histology preparations (OIE 2000a).

M.2.3.2 *Confirmatory*

M.2.3.2.1 Histopathology (Level II)

It is recommended that at least two dorso-ventral sections through the cardiac cavity, gonad and gills of oysters over 18 months – 2 years (> 30 mm shell height) be examined for screening purposes. These sections should be fixed immediately in a fast fixative such as 1G4F. Fixatives such as Davidsons or 10% seawater buffered formalin may be used for whole oysters (see M.1.3.3.3), but these do not allow serial tissue sections to be collected for subsequent confirmatory Electron Microscopy (EM) diagnosis, if required. Davidson's fixative is recommended for subsequent PCR-based confirmation techniques.

Several standard stains (*e.g.*, haematoxylin-eosin) enable detection of *Bonamia* spp.. The parasites measure 2-5 µm and occur within the haemocytes or epithelia (as described above) or, more rarely, loose within the haemolymph or gut/mantle lumens.

M.2.4 **Diagnostic Methods**

More detailed methods for diagnosis can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.2.4.1 *Presumptive*

M.2.4.1.1 Histopathology and Cytology (Level II)

Histology and cytology (Level II), as described under M.2.3.2.1, may be used. For first-time diagnoses, a back up tissue specimen fixed for EM is recommended (M.2.4.2.1).

M.2.4. 2 *Confirmatory*

M.2.4.2.1 Transmission Electron Microscopy (TEM) (Level III)

Tissue for TEM can be fixed in 1G4F (M.1.3.3.3), however, where it is likely that TEM will be required for confirmatory diagnosis (M.2.4.1.1), small (< 1 mm cubed) sub-samples of infected tissue should be fixed in 2-3% buffered glutaraldehyde prepared with ambient salinity filtered seawater. Fixation should not exceed 1 hr. Longer storage in gluteraldehyde fixative is possible, however membrane artifacts can be produced. Tissues should be rinsed in a suitable buffer prior to post-fixing in 1-2% osmium tetroxide (= osmic acid - *highly toxic*). This post-fixative must also be rinsed with buffered filtered (0.22 µm) seawater prior to dehydration and resin-embedding.

Post-fixed tissues should be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides and stained with Toluidine Blue is one method of selecting the tissue specimens for optimum evidence of putative *Bonamia* spp. Ultrathin sections are then mounted on copper grids (with or without formvar coating) for staining with lead citrate + uranyl acetate or equivalent EM stain.

Ultrastructural differences between *B. ostreae* and *Bonamia* sp. include diameter (*B. ostreae* = 2.4 ± 0.5 µm; *Bonamia* sp. = 2.8 ± 0.4 µm in *O. angasi,* 3.0 ± 0.3 µm in *T. chilensis*); mean number of mitochondrial profiles/section (*B. ostreae* = 2 ± 1; *Bonamia* sp. = 4 ± 1 in *O. angasi,* 3 ±1 in *T. chilensis*), mean number of haplosporosomes/section (*B. ostreae* = 7 ± 5;

M.2 Bonamiosis (*Bonamia* sp., *B. ostreae*)

Bonamia sp. = 10 ± 4 in *O. angasi,* 14 ± 6 in *T. chilensis*); percentage of sections containing large lipid globules (*B. ostreae* = 7%; *Bonamia* sp. in *O. angasi* = 30%; in *T. chilensis* = 49%), large lipid globules/section (*B. ostreae* = 0.3 ± 0.6; *Bonamia* sp. = 0.5 ± 0.8 in *O. angasi,* 0.8 ± 0.9 in *T. chilensis*). Both species are distinguished from *Mikrocytos* spp. by having a centrally-placed nucleus.

Plasmodial forms of *Bonamia* sp. in *T. chilensis* are distinguished from *Bonamia ostreae* by their size (4.0 -4.5 μm diameter), irregular cell and nucleus profile, amorphous cytoplasmic inclusions (multi-vesicular bodies) and arrays of Golgi-like smooth endoplasmic reticula. Other developmental stages are more electron dense and smaller in diameter (3.0 -3.5 μm).

M.2.5 **Modes of Transmission**

Prevalence and intensity of infection tends to increase during the warm water season with peaks in mortality in September/October in the northern hemisphere, and January to April, in the southern hemisphere. The parasite is difficult to detect prior to the proliferation stage of development or in survivors of an epizootic. Cohabitation and tissue homogenate/haemolymph inoculations can precipitate infections indicating that transmission is direct (no intermediate hosts are required). There is a pre-patent period of 3-5 months between exposure and appearance of clinical signs of *B. ostreae* infection. In New Zealand the pre-patent period for *Bonamia* sp. infection may be as little as 2.5 months and rarely exceeds 4 months.

M.2.6 **Control Measures**

None known. Reduced stocking densities and lower water temperatures appear to suppress clinical manifestation of the disease, however, no successful eradication procedures have worked to date. Prevention of introduction or transfer of oysters from *Bonamia* spp. enzootic waters into historically uninfected waters is recommended.

M.2.7 **Selected References**

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M.3 MARTEILIOSIS (*MARTEILIA REFRINGENS*, *M. SYDNEYI*)

M.3.1 Background Information

M.3.1.1 *Causative Agents*

Marteiliosis is caused by two species of parasites, belonging to the Phylum Paramyxea. *Marteilia refringens* is responsible for Aber Disease (*a.k.a* Digestive Gland Disease) of European oysters (*Ostrea edulis*) and *Marteilia sydneyi* is responsible for QX Disease of *Saccostrea glomerata* (syn. *Crassostrea commercialis, Saccostrea commercialis*) and, possibly, *Saccostrea echinata*. More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.3.1.2 *Host Range*

Ostrea edulis is infected by *Marteilia refringens.* Other host species include *Tiostrea chilensis*, *Ostrea angasi*, *O. puelchana*, *Cerastoderma* (= *Cardium*) *edule*, *Mytilus edulis*, *M. galloprovincialis*, *Crassostrea gigas* and *C. virginica*. *Marteilia sydneyi* infects *Saccostrea glomerata* and possibly *S. echinata*. Another marteiliad, *Marteilia maurini*, infects mussels (*Mytilus edulis* and *M. galloprovincialis*) from France, Spain and Italy. This species is not readily distinguished morphologically from *M. refringens* and distinct species status is under investigation. An unidentified marteiliad was responsible for mass mortalities of the Calico scallop (*Argopecten gibbus*) in Florida in the late 1980's, but has not re-appeared since. Another *Marteilia*-like species was reported from the giant clam, *Tridacna maxima*. Other species of *Marteilia* that have been described include *M. lengehi* from *Saccostrea* (*Crassostrea*) *cucullata* (Persian Gulf and north Western Australia) and *M. christenseni* in *Scrobicularia plana* (France). These are differentiated from *M. refringens and M. sydneyi* by the cytoplasmic contents of the sporangia and spore morphology.

M.3.1.3 *Geographic Distribution*

Marteilia refringens is found in *O. edulis* in southern England, France, Italy, Portugal, Spain, Morocco and Greece. *Marteilia sydneyi* is found in *S. glomerata* in Australia (New South Wales, Queensland and Western Australia).

M.3.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

No positive report of the disease in any country for the 2 year reporting periods. Most countries have no information about the occurrence of the disease (OIE 1999,OIE 2000b).

M.3.2 Clinical Aspects

Early stages of *Marteilia refringens* develop in the digestive ducts, intestinal and stomach epithelia and gills (Fig.M.3.2a). Later, spore-forming stages appear in the blind-ending digestive tubule epithelia (Fig.M.3.2b). Proliferation of the parasite is associated with emaciation and exhaustion of glycogen reserves, gross discolouration of the digestive gland, cessation of feeding and weakening. Mortalities appear to be associated with sporulation of the parasite and disruption of the digestive tubule epithelia.

M.3.3 Screening Methods

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.3.3.1 *Presumptive*

M.3.3.1.1 Gross Observations (Level I)

Slowed growth, gaping and mortalities of *Ostrea edulis* and other susceptible species should be considered suspect for Marteiliosis. Gross signs are not specific for Bonamiosis or Marteiliosis and require Level II examination.

M.3.3.1.2 Tissue Imprints (Level II)

Cut a cross-section through the digestive gland, blot away excess water with blotting paper and dab the cut section of the digestive gland onto a clean microscope slide. Fix tissue imprint for 2-3 min in 70% methanol. Quick and effective staining can be achieved with a commercially available blood-staining (cytological) kit, using the manufacturer's instructions. The stained slides are then rinsed (gently) under tap water, allowed to dry and cover-slipped using a synthetic resin mounting medium.

The parasite morphology is as described for histology (M.3.3.2.1), although colouration may vary with the stain chosen. Initial screening with a haematoxylin or trichrome stain, as used for his-

tology, may assist familiarisation with tissue imprint characteristics prior to using a dip-quick method. An observation time of 10 mins at 10-25x magnification is considered sufficient for screening purposes.

M.3.3.2 *Confirmatory*

M.3.3.2.1 Histopathology (Level II)

Two dorso-ventral tissue section (2-3 mm thick) are recommended for screening purposes. These can be removed from oysters over 18-24 months old (or >30 mm shell height) for immediate fixation in a fast fixative, such as 1G4F. Davidsons or 10% buffered formalin may be used for larger samples or whole oysters (see M.1.3.3.3) but these provide less satisfactory results if subsequent processing for Transmission Electron Microscopy (TEM) (M.3.4.2.1) is required (e.g., for species identification). Several standard stains (*e.g.*, haematoxylin-eosin) enable detection of *Marteilia* spp..

The early stages of development occur in the stomach, intestine and digestive duct epithelia (usually in the apical portion of the cell) and appear as basophilic, granular, spherical inclusions (Fig.M.3.2a). Later stages occur in the digestive tubules, where sporulation may induce hypertrophy of the infected cell. *Marteilia* spp. spores contain eosinophilic, "refringent", bodies which are easily detected at 10-25 x magnification under light microscopy (Fig.M.3.2b).

(SE McGladdery)

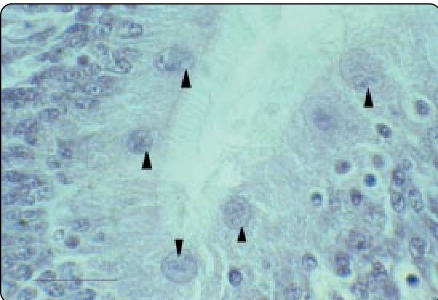

Fig.M.3.2a. Digestive duct of a European oyster, *Ostrea edulis*, showing infection of distal portion of the epithelial cells by plasmodia (arrows) of *Marteilia refringens*. Scale bar 15 µm (H&E).

(SE McGladdery)

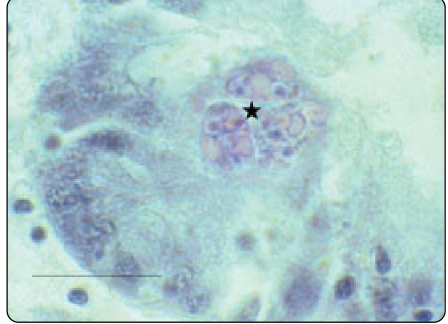

Fig.M.3.2b. Digestive tubule of a European oyster, *Ostrea edulis*, showing refringent spore stage of *Marteilia refringens* (star). Scale bar 50 µm (H&E).

(RD Adlard)

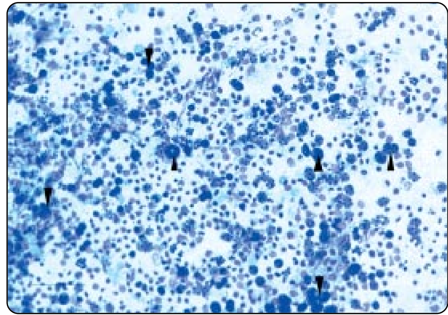

Fig.M.3.4.1.1a. Tissue imprint from *Saccostrea commercialis* (Sydney rock oyster) heavily infected by *Marteilia sydneyi* (arrows) (QX disease). Scale bar 250 µm (H&E).

(RD Adlard)

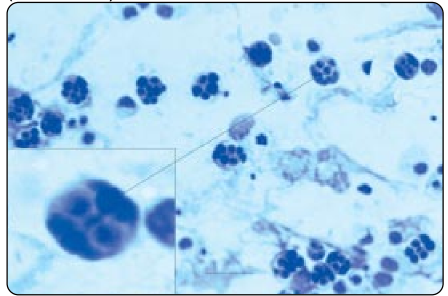

Fig.M.3.4.1.1b. Oil immersion of tissue squash preparation of spore stages of *Marteilia sydneyi* from Sydney rock oyster (*Saccostrea commercialis*) with magnified inset showing two spores within the sporangium. Scale bar 50 µm (H&E).

M.3 Marteiliosis (*Marteilia refringens*, *M. sydneyi*)

M.3.4 **Diagnostic Methods**

More detailed methods for diagnosis can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.3.4.1 *Presumptive*

M.3.4.1.1 Tissue Imprints (Level II)

As described under M.3.3.1.2, tissue imprints may also be used for presumptive diagnoses (Fig.M.3.4.1.1a,b). For first-time diagnoses back up tissues should be fixed for histology and EM confirmatory diagnosis.

M.3.4.1.2 Histopathology (Level II)

Histology techniques as described under M.3.3.2.1, may be used. For first-time diagnoses a back up tissue specimen fixed for EM is recommended, as described below.

M.3.4.2 *Confirmatory*

M.3.4.2.1 Transmission Electron Microscopy (TEM) (Level III)

TEM tissue preparation involves fixing tissues either in 1G4F (M.1.3.3.3) or small (< 1 mm cubed) sub-samples of infected tissue in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater. Ideally, fixation in 2-3% gluteraldehyde should not exceed 1 hr, since longer storage may induce membranous artifacts. Tissues should be fixed in 1G4F for 12-24 hrs. Following primary fixation, rinse tissues in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO_4 = osmic acid - *highly toxic*). Secondary fixation should be complete within 1 hr. The OsO_4 fixative must also be rinsed with buffer/filtered (0.22 µm) seawater prior to dehydration and resin-embedding.

Post-fixed tissues can be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 µm sections melted onto glass microscope slides with 1% toluidine blue solution is one method of selecting the tissue specimens for optimum evidence of possible *Marteilia* spp. Ultrathin sections are then mounted on copper grids (with or without formvar coating), and stained with lead citrate + uranyl acetate (or equivalent EM stain).

Marteilia refringens plasmodia contain striated inclusions, eight sporangial primordia, with up to four spores to each mature sporangium. *Marteilia sydneyi* has a thick layer of concentric membranes surrounding the mature spore, lacks striated inclusions in the plasmodia, forms eight to sixteen sporangial primordia in each plasmodium and each sporangium contains two (rarely three) spores.

M.3.4.2.2 *In situ* Hybridization (Level III)

In situ hybridization (Level III) techniques are under development but not yet available commercially. Information on the current status of these and related molecular probe techniques may be obtained from IFREMER Laboratory at La Tremblade, France (OIE 2000a, Annex MAI).

M.3.5 **Modes of Transmission**

Marteilia refringens transmission appears to be restricted to periods when water temperatures exceed 17°C. High salinities may impede *Marteilia* spp. multiplication within the host tissues. *Marteilia sydneyi* also has a seasonal period of transmission with infections occurring generally from mid- to late-summer (January to March). Heavy mortalities and sporulation occur all year round. The route of infection and life-cycle outside the mollusc host are unknown. Since it has not been possible to transmit the infection experimentally in the laboratory, an intermediate host is suspected. This is reinforced by recent observations showing spores do not survive more than 7-10 days once isolated from the oyster. Cold temperatures prolong survival (35 days at 15°C). Spore survival within fish or birds was limited to 2 hrs, suggesting they are an unlikely mode of dispersal or transmission.

M.3.6 **Control Measures**

None known. High salinities appear to suppress clinical manifestation of the disease, however, no eradication attempts have been successful, to date. Prevention of introduction or transfer of oysters or mussels from *Marteilia* spp. enzootic waters into historically uninfected waters is recommended.

M.3.7 **Selected References**

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M.3 Marteiliosis (*Marteilia refringens*, *M. sydneyi*)

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M.4 MIKROCYTOSIS (*MIKROCYTOS MACKINI*, *M. ROUGHLEYI*)

M.4.1 **Background Information**

M.4.1.1 *Causative Agents*

Mikrocytosis is caused by two species of parasites of uncertain taxonomic affinity. *Mikrocytos mackini* is reponsible for Denman Island Disease (Microcell disease) of Pacific oysters (*Crassostrea gigas*), and *Mikrocytos roughleyi* is responsible for Australian Winter Disease (Winter Disease, Microcell Disease) of Sydney rock oysters, *Saccostrea glomerata.* More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.4.1.2 *Host Range*

Mikrocytos mackini naturally infects *Crassostrea gigas* (Pacific oysters). *Ostrea edulis* (European oysters), *O. conchaphila* (= *O. lurida*) (Olympia oyster) and *Crassostrea virginica* (American oysters) growing in enzootic waters are also susceptible to infection. *Saccostrea glomerata* (*Crassostrea commercialis*, *Saccostrea commercialis*) (Sydney rock oyster) is the only known host for *Mikrocytos roughleyi*.

M.4.1.3 *Geographic Distribution*

Mikrocytos mackini is restricted to specific localities around Vancouver Island and southwest coast of the Pacific coast of Canada. The parasite is limited to waters with temperatures below 12?C. *Mikrocytos roughleyi* occurs in central to southern New South Wales, and at Albany and Carnarvon, Western Australia.

M.4.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

No positive report during the reporting periods for 1999 and 2000. In Australia, last year of occurrence was 1996 (in New South Wales and Western Australia. Most countries have no information about occurrence of the disease (OIE 1999, OIE 2000b).

M.4.2 **Clinical Aspects**

Mikrocytos mackini initiates focal infections of the vesicular connective tissue cells. This elicits haemocyte infiltration and abscess formation. Grossly visible pustules (Fig.M.4.2a), abscess lesions and tissue ulcers, mainly in the mantle, may correspond to brown scar formation on the adjacent surface of the inner shell. However, such lesions are not always present. Small cells, 1-3 µm in diameter, are found (rarely) around the periphery of advanced lesions, or in connective tissue cells in earlier stages of disease development. Severe infections appear to be restricted to oysters over 2 years old.

Mikrocytos roughleyi induces a systemic intracellular infection of the haemocytes (never the connective tissue cells) which may result in focal lesions in the gills, connective, gonadal and digestive tract.

M.4.3 **Screening Methods**

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.4.3.1 *Presumptive*

M.4.3.1.1 Gross Observations (Level I)

Slowed growth, gaping and mortalities of *Crassostrea gigas* and *Saccostrea glomerata* should be considered suspect for Mikrocytosis. Gross signs are not pathogen specific and require Level II examination, at least for first-time observations.

M.4.3.1.2 Cytological Examination and Tissue Imprints (Level II)

Heart impressions (dabs) can be made onto a clean microscope slide and air-dried. Once dry, the slide is fixed in 70% methanol. Quick and effective staining can be achieved with commercially available blood-staining (cytological) kits, using the manufacturer's instructions. The stained slides are then rinsed (gently) under tap water, allowed to dry and cover-slipped using a synthetic resin mounting medium. Intracytoplasmic parasites in the haemocytes will match the descriptions given above for histology. This technique is more applicable to *M. roughleyi* than *M. mackini*.

Tissue sections through mantle tissues (especially abscess/ulcer lesions, where present) are cut and excess water removed with blotting paper. The cut section is dabbed onto a clean microscope slide, fixed for 2-3 minutes in 70% methanol and stained. Quick and effective staining can be achieved using a commercially available blood-staining (cytological) kits, using the manufacturer's instructions. The stained slides

M.4 MIKROCYTOSIS (*Mikrocytos mackini*, *M. roughleyi*)

(SM Bower)

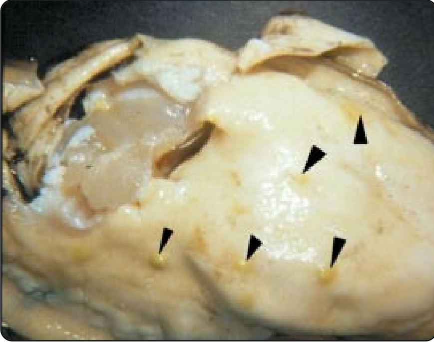

Fig.M.4.2a. Gross abscess lesions (arrows) in the mantle tissues of a Pacific oyster (*Crassostrea virginica*) severely infected by *Mikrocytos mackini* (Denman Island Disease).

(SM Bower)

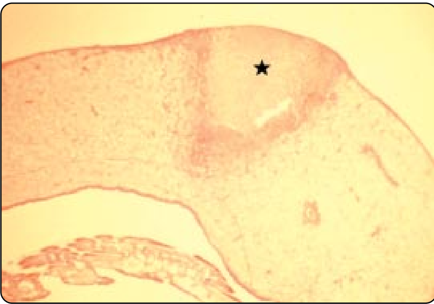

Fig.M.4.3.2.1a. Histological section through mantle tissue abscess corresponding to the gross lesions pictured in Fig.M.4.2a, in a Pacific oyster (*Crassostrea gigas*) infected by *Mikrocytos mackini* (H&E).

(SM Bower)

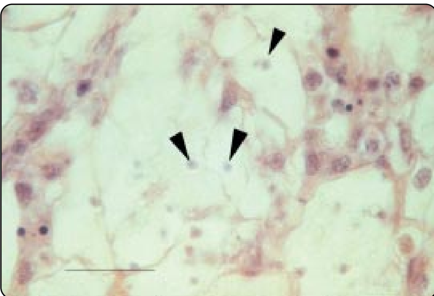

Fig.M.4.3.2.1b. Oil immersion of *Mikrocytos mackini* (arrows) in the connective tissue surrounding the abscess lesion pictured in Fig.M.4.3.2.1a. Scale bar 20 µm (H&E).

are then rinsed (gently) under tap water, allowed to dry and cover-slipped using a synthetic resin mounting medium.

The parasite morphology is as described for histology (M.4.3.2.1), although colouration may vary with the stain chosen. Initial screening with a haematoxylin or trichrome stain, as used for histology, may assist familiarisation with tissue imprint characteristics prior to using a dip-quick method. An observation time of 10 mins under oil immersion is considered sufficient for screening purposes.

M.4.3.2 *Confirmatory*

M.4.3.2.1 Histopathology (Level II)

It is recommended that at least two dorso-ventral sections (2-3 mm) through each oyster be examined using oil immersion for screening purposes. Sections from oysters >2 yrs (or >30 mm shell height) should be fixed immediately in a fast fixative such as 1G4F. Davidsons or 10% buffered formalin may be used for smaller or whole oysters (see M.1.3.3.3) but these fixatives are not optimal for subsequent confirmatory Electron Microscopy (EM) diagnosis (M.4.4.2.1), or species identification, if required. Also smaller oysters are not the recommended size-group for *Mikrocytos* screening. Sections through pustules, abscess or ulcer lesions should selected where present. Several standard stains (*e.g.*, haematoxylin-eosin) enable detection of *Mikrocytos* spp.

Mikrocytos mackini appears as 2-3 µm intracellular inclusions in the cytoplasm of the vesicular connective tissues immediately adjacent to the abscess-like lesions (Fig. M.4.3.2.1a,b). It may also be observed in muscle cells and, occasionally, in haemocytes or free, within the lesions. It is distinguished from *Bonamia* by an eccentric nucleus and from *M. roughleyi* by the consistent absence of a cytoplasmic vacuole, and the presence of a mitochondrion in *M. roughleyi*. These features will not be clear under oil and require confirmation using 1 micron resin sections or TEM (described below). Neither of these techniques, however, are practical for screening purposes.

Mikrocytos roughleyi measures 1-3 µm in diameter and occurs exclusively in the haemocytes. A cytoplasmic vacuole may or may not be present. When present, it displaces the nucleus peripherally. Nucleolar structures may or may not be visible under oil immersion resolution for this intracellular parasite.

M.4.4 Diagnostic Methods

More detailed methods for diagnosis can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.4.4.1 *Presumptive*

M.4.4.1.1 Histopathology and Tissue Imprints (Level II)

Histology (M.4.3.2.1) may be used, however, for first-time diagnoses, EM confirmation is recommended (M.4.4.2.2). Tissue imprints may also be used for presumptive diagnoses, where they demonstrate the features described under M.4.3.1.2.

M.4.4.2 *Confirmatory*

M.4.4.2.1 Histopathology and Tissue Imprints (Level II)

Histology (M.4.3.2.1) and tissue imprints (M.4.3.1.2) may be used, however, for first-time diagnoses, EM confirmation is recommended (M.4.4.2.2).

M.4.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

Tissues should be fixed in 1G4F for 12-24 hours. Following primary fixation, rinse tissues in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO_4 = osmic acid - *highly toxic*). Secondary fixation should be complete within 1 hour. The OsO_4 fixative must also be rinsed with buffer/filtered (0.22 microns) seawater prior to dehydration and resin-embedding.

Post-fixed tissues can be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides with 1% toludine blue solution is one method of selecting the tissue specimens for optimum evidence of putative *Mikrocytos* spp. Ultrathin sections are then mounted on copper grids (with or without formvar coating), and stained with lead citrate + uranyl acetate (or equivalent EM stain).

Mikrocytos mackini is distinguished ultrastructurally (as well as by tissue location and host species) from *Bonamia* spp. by the location of the nucleolus. In *M. mackini* it is in the centre of the nucleus, while in *B. ostreae* it is eccentric. *Mikrocytos mackini* also lacks mitochondria. The ultrastructural characteristics of *Mikrocytos roughleyi* have not been published, however, it is distinguished from *M. mackini* by the presence of a cytoplasmic vacuole (along with completely different geographic, host and tissue locations!).

M.4.5 Modes of Transmission

Mikrocytos mackini transmission appears restricted to early spring (April-May) following periods of 3-4 months at water temperatures < 10°C. High salinities (30-35 ppt) appear to favour parasite proliferation and mortalities of approximately 40% occur in sub-tidal or low-tide populations of older oysters.

Mikrocytos roughleyi is also associated with low temperatures and high salinities killing up to 70% of mature Sydney rock oysters in their third winter before marketing. This usually follows a prepatent (sub-clinical) period of approximately 2.5 months.

Transmission of *M. mackini* has been achieved by exposure of susceptible oysters to homogenates from infected oysters as well as to proximal exposure, thus, it is believed that this species has a direct life-cycle. *M. roughleyi* is also thought to be transmitted directly from oyster to oyster.

M.4.6 Control Measures

Circumvention of mortalities has been achieved for *M. mackini* at enzootic sites by relaying oysters to high tide levels during the peak transmission period in April-May to reduce exposure to the water-borne infectious stages. No control measures are known for *M. roughleyi.*

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M.5 PERKINSOSIS (*PERKINSUS MARINUS*, *P. OLSENI*)

M.5.1 **Background Information**

M.5.1.1 *Causative Agents*

Perkinsosis is caused by two species of protistan parasite belonging to the phylum Apicomplexa (although recent nucleic acid investigations suggest a possible affiliation with the dinoflagellates). *Perkinsus marinus* is responsible for "Dermo" disease in *Crassostrea virginica* (American oysters) and *Perkinus olseni* causes perkinsosis in many bivalve species in tropical and subtropical waters. Other perkinsiid species are known to infect clams in Europe (*Perkinsus atlanticus*) and the eastern USA (*Perkinsus* spp.), as well as Japanese (Yesso) scallops, *Patinopecten yessoensis* in Pacific Canada (*Perkinsus qugwadi*). The taxonomic relationship between these and the two species listed as 'notifiable' by OIE is currently under investigation. More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.5.1.2 *Host Range*

Perkinsus marinus (formerly known as *Dermocystidium marinum* and *Labyrinthomyxa marinus*) infects *Crassostrea virginica* (American oysters). Experimental infection to *C. gigas* (Pacific oysters) is possible, but they appear more resistant than *C. virginica. Perkinsus olseni* shows a strong rDNA similarity to *Perkinsus altanticus* of *Ruditapes decussatus* and the speciation within this genus, as mentioned under M.5.1.1, is currently under nucleic acid investigation. Recognised hosts of *P. olseni* are the abalone species: *Haliotis rubra, H. cyclobates, H. scalaris* and *H. laevigata*. More than 50 other molluscan species harbour *Perkinsus* spp., as well as other possibly related species, without apparent harmful effects (*e.g.,* in *Arca* clams [Fig.M.5.1.2a] and *Pinctada* pearl oysters [Fig.M.5.1.2b]).

M.5.1.3 *Geographic Distribution*

Perkinsus marinus is found along the east coast of the United States from Massachusetts to Florida, along the Gulf of Mexico coast to Venezuela, and in Puerto Rico, Cuba and Brazil. It has also been introduced into Pearl Harbour, Hawaii. Range extension into Delaware Bay, New Jersey, Cape Cod and Maine are attributed to repeated oyster introductions and increased winter water temperatures. *Perkinsus olseni* occurs in South Australia. Other species occur in Atlantic and Pacific oceans and the Mediterranean Sea.

M.5.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

P. marinus was not reported in Australia during 1999 and 2000 reporting periods; *P. olseni* likewise not reported in 1999 and 2000 (last year of occurrence in South Australia in 1997, and in 1995 in New South Wales and Western Australia). Suspected in Korea RO for reporting period 1999 and 2000. In New Zealand, positively reported from April to December 2000. *Perkinsus olseni* occurs in wild populations of New Zealand cockles, *Austrovenus stutchburyi* (Family Veneridae) and two other bivalve species, *Macomona liliana* (Family Tellinidae) and Barbatia novae-zelandiae (Family Arcidae). These species occur widely in the coast of New Zealand. Affected locations have been the Waitemata and Kaipara Harbours but the organism is probably enzootic in the warmer waters of northern New Zealand (OIE 1999, OIE 2000a).

M.5.2 **Clinical Aspects**

The effects of *Perkinsus marinus* on *Crassostrea virginica* range from pale appearance of the digestive gland, reduced condition indices, severe emaciation, gaping, mantle retraction, retarded gonadal development and growth and occasional abscess lesions. Mortalities of up to 95% have occurred in infected *C. virginica* stocks.

Proliferation of *Perkinsus olseni* results in disruption of connective and epithelial tissues and some host species show occasional abscess formation. Pustules up to 8 mm in diameter in affected *Haliotis* spp. reduce market value and have been associated with heavy losses in *H. laevi gata*.

M.5.3 **Screening Methods**

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.5.3.1 *Presumptive*

M.5.3.1.1 Gross Observations (Level I)

Slowed growth, gaping and mortalities of *Crassostrea virginica* and *Haliotis* spp., as well as other mollusc species in *Perkinsus*-enzootic waters should be considered suspect for Perkinsiosis. Gross signs are not pathogen-specific and require Level II examination, at least for first time observations.

M.5 Perkinsosis (*Perkinsus marinus*, *P. olseni*)

(PM Hine)

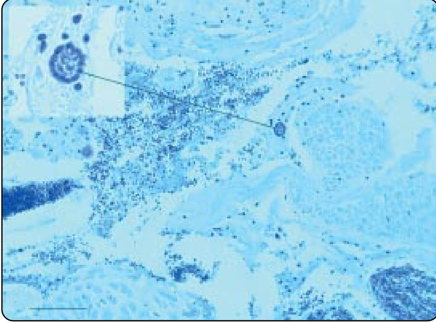

Fig.M.5.1.2a. *Arca* clam showing a *Perkinsus*-like parasite within the connective tissue. Magnified insert shows details of an advanced 'schizont' like stage with trophozoites showing vacuole-like inclusions. Scale bar 100 µm. (H&E).

(PM Hine)

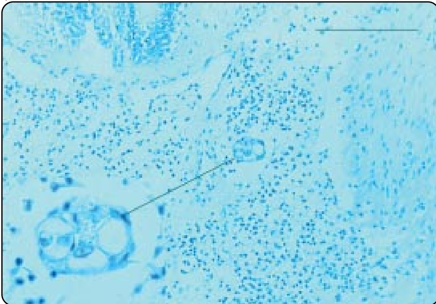

Fig.M.5.1.2b. *Pinctada albicans* pearl oyster showing a *Perkinsus*-like parasite. Magnified insert shows details of a 'schizont'-like stage containing 'trophozoites' with vacuole-like inclusions. Scale bar 250 µm (H&E).

(SM Bower)

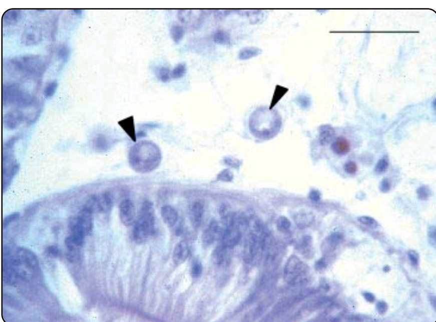

Fig.M.5.3.2.1a. Trophozoite ('signet-ring') stages of *Perkinsus marinus* (arrows), the cause of 'Dermo' disease in American oyster (*Crassostrea virginica*) connective tissue. Scale bar 20 µm (H&E).

(SE McGladdery)

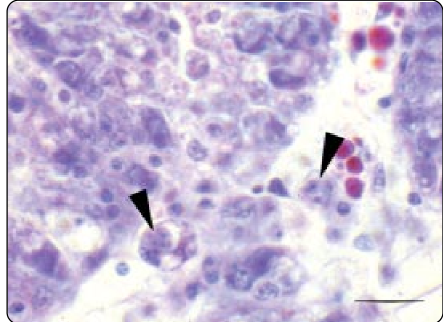

Fig.M.5.3.2.1b. Schizont ('rosette') stages of *Perkinsus marinus* (arrows), the cause of 'Dermo' disease in American oyster (*Crassostrea virginica*) digestive gland connective tissue. Scale bar 30 µm (H&E).

(SE McGladdery)

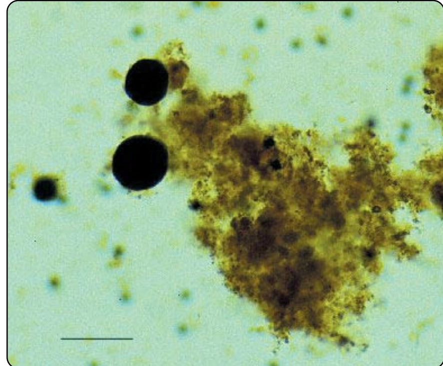

Fig.5.3.2.2. Enlarged hypnospores of *Perkinsus marinus* stained blue-black with Lugol's iodine following incubation in fluid thioglycollate medium. Scale bar 200 µm.

M.5.3.2 *Confirmatory*

M.5.3.2.1 Histopathology (Level II)

It is recommended that at least two dorso-ventral sections through each oyster be examined using oil immersion for screening purposes. Sections from oysters >2 yrs (or >30 mm shell height) should be fixed immediately in a fast fixative such as 1G4F. Davidson's or 10% buffered formalin may be used for smaller or whole oysters (see M.1.3.3.3) but these fixatives are not optimal for subsequent confirmatory Electron Microscopy (EM) diagnosis (M.5.4.2.1), or species identification, if required. Sections through pustules, abscess or ulcer lesions should be selected, where present. Several standard stains (*e.g.*,

haematoxylin-eosin) enable detection of *Perkinsus* spp.

Perkinsus marinus infections are usually systemic, with trophozoites occuring in the connective tissue of all organs. Immature trophozoites (meronts, merozoites or aplanospores) measure 2-3 µm in diameter. "Signet-ring" stages are mature trophozoites, measuring 3-10 µm in diameter, each with a visible eccentric vacuole displacing the nucleus and cytoplasm peripherally (Fig.M.5.3.2.1a). The "rosette" stage (tomonts, sporangia or schizonts) measure 4-15 µm in diameter and can contain 2, 4, 8, 16 or 32 developing trophozoites (Fig.M.5.3.2.1b).

Perkinsus olseni shows the same developmental stages although the trophozoite stages are larger ranging from 13-16 µm in diameter. Due to host and parasite diversity, however, morphological features cannot be considered specific.

M.5.3.2.2 Fluid Thioglycollate Culture (Level II)

Tissue samples measuring 5-10 mm are excised (select lesions, rectal and gill tissues) and placed in fluid thioglycollate medium containing antibiotics. Incubation temperature and time varies per host species and environment. The standard protocol for *P. marinus* is 22-25°C for 4-7 days in the dark. Warmer temperatures can be used for *P. olseni*.

The cultured parasites expand in size to 70-250 ?m in diameter. Following incubation, the tissues and placed in a solution of 1:5 Lugol's iodine:water for 10 minutes. The tissue is then teased apart on a microscope slide and examined, using low power on a light microscope, for enlarged hypnospores with walls that stain blue-black (Fig.M.5.3.2.2).

M.5.4 **Diagnostic Methods**

More detailed methods for diagnostics can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.5.4.1 *Presumptive*

M.5.4.1.1 Histopathology (Level II)

Histology (M.5.3.2.1), may be used. However, for first-time diagnoses a back up tissue specimen fixed for EM is recommended (M.5.4.2.1).

M.5.4.1.2 Fluid Thioglycollate Medium Culture (Level II)

Fluid thioglycollate medium culture (Level II) may also be used for presumptive diagnosis (M.5.3.2.2).

M.5.4.2 *Confirmatory*

M.5.4.2.1 Transmission Electron Microscopy (TEM) (Level III)

TEM is required to detect the species-specific ultrastructure of the zoospore stage of development (collected from zoospore release from cultured aplanospores [prezoosporangia]). Tissue preparation involves fixing concentrated zoospores or zoosporangia (produced by placing the aplanospores into filtered ambient seawater, where they develop into zoosporangia and produce hundreds of motile zoospores) in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater. Oyster tissues can also be fixed in 1G4F for 12-24 hrs. Following primary fixation, rinse tissues in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO_4 = osmic acid - *highly toxic*). Secondary fixation should be complete within 1 hr. The OsO_4 fixative must also be rinsed with buffer/filtered (0.22 µm) seawater prior to dehydration and resin-embedding.

Post-fixed tissues can be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 ?m-thick sections melted onto glass microscope slides with 1% toluidine blue solution is one method of selecting the tissue specimens for optimum evidence of putative *Perkinsus* spp. Such pre-screening should not be necessary for concentrated zoospore or zoosporangia preparations. Ultrathin sections are then mounted on copper grids (with or without formvar coating), and stained with lead citrate + uranyl acetate (or equivalent EM stain).

The anterior flagellum of *Perkinsus marinus* zoospores is ornamented with a row of hair- and spur-like structures. The posterior flagellum is smooth. An apical complex is present, consisting of a conoid, sub-pellicular microtubules, rhoptries, rectilinear micronemes and conoid-associated micronemes. Large vacuoles are also present at the anterior end of the zoospore.

M.5.5 **Modes of Transmission**

Proliferation of *Perkinsus* spp. is correlated with warm water temperatures (>20°C) and this coincides with increased clinical signs and mortalities. Effects appear cumulative with mortalities peaking at the end of the warm water season in each hemisphere. The infective stage is a biflagellate zoospore which transforms into the feeding trophozoite stage after entering the host's tissues. These multiply by binary fission within the host tissues. *Perkinsus marinus* shows a wide salinity tolerance range. *Perkinsus olseni* is associated with full strength salinity environments.

Direct transmission of *Perkinsus* spp. has been demonstrated by exposure of susceptible hosts to infected hosts, including cross-species transmission for *P. olseni*. There is currently no evidence of cross-genus transmission of *P. marinus*.

M.5.6 **Control Measures**

None known for *Perkinsus* spp. Most efforts against *P. marinus* have concentrated on development of resistant (tolerant) stocks of oysters. These currently show potential for surviving in enzootic areas, but are not recommended for use in non-enzootic areas due to their potential to act as sub-clinical carriers of the pathogen. Some success has been achieved, however, in preventing *P. marinus* infection of hatchery-reared larval and juvenile oysters using filtration and UV sterilization of influent water. The almost ubiquitous occurrence of *Perkinsus* in many bivalve species around mainland Australia makes control by restriction of movements impractical.

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M.6 HAPLOSPORIDIOSIS (*HAPLOSPORIDIUM COSTALE*, *H. NELSONI*)

M.6.1 **Background Information**

M.6.1.1 *Causative Agents*

Haplosporidiosis is caused by two species of protistan parasite belonging to the phylum Haplosporidia. *Haplosporidium nelsoni* (syn. *Minchinia nelsoni*) is responsible for "MSX" (multinucleate sphere X) disease in *Crassostrea virginica* (American oysters) and *Haplosporidium costale* (*Minchinia costale*) causes "SSO" (seaside organism) disease in the same species. More information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

M.6.1.2 *Host Range*

Both *Haplosporidium nelsoni* and *H. costale* cause disease in *Crassostrea virginica* (American oysters). Recently a *Haplosporidium* sp. from *Crassostrea gigas* (Pacific oyster) has been identified as *H. nelsoni* using DNA sequencing of small sub-unit ribosomal DNA.

M.6.1.3 *Geographic Distribution*

Haplosporidium nelsoni occurs in American oysters along the Atlantic coast of the United States from northern Florida to Maine. Enzootic areas appear limited to Delaware Bay, Chesapeake Bay, Long Island Sound and Cape Cod. *Haplosporidium nelsoni* has been found in *C. gigas* from California and Washington on the Pacific coast of the USA, and Korea, Japan and France.

(PM Hine)

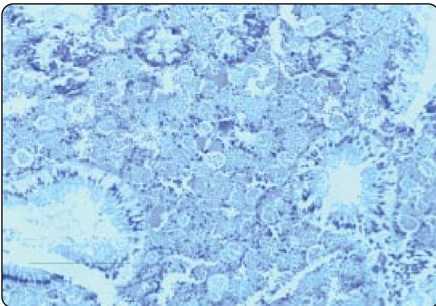

Fig.M.6.1.3a. Massive connective tissue and digestive tubule infection by an unidentified *Haplosporidium*-like parasite in the gold-lipped pearl oyster *Pinctada maxima* from north Western Australia. Scale bar 0.5 mm (H&E).

(PM Hine)

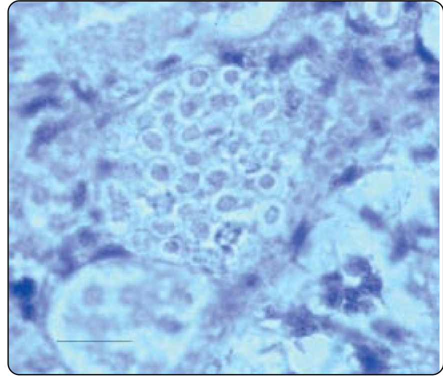

Fig.M.6.1.3b. Oil immersion magnification of the operculated spore stage of the *Haplosporidium*-like parasite in the gold-lipped pearl oyster *Pinctada maxima* from north Western Australia. Scale bar 10 µm. (H&E).

(PM Hine)

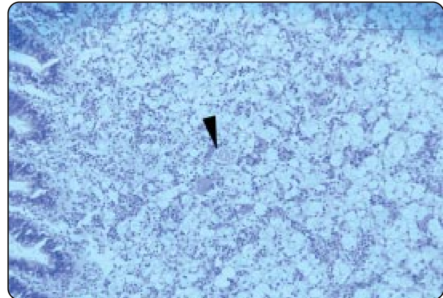

Fig.M.6.1.3c. Haemocyte infiltration activity in the connective tissue of a Sydney rock oyster (*Saccostrea cucullata*) containing spores of a *Haplosporidium*-like parasite (arrow). Scale bar 0.5 mm. (H&E).

(PM Hine)

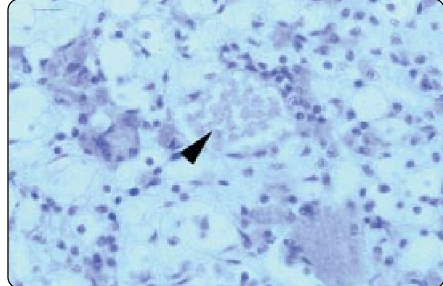

Fig.M.6.1.3d. Oil immersion magnification of *Haplosporidium*-like spores (arrow) associated with heavy haemocyte infiltration in a Sydney rock oyster (*Saccostrea cucullata*). Scale bar 10 µm. (H&E).

M.6 Haplosporidiosis (*Haplosporidium costale*, *H. nelsoni*)

Haplosporidium costale has been reported solely from *C. virginica* from the Atlantic coast of the United States and has a small subunit rDNA distinct from that of *H. nelsoni*. *Hapolosporidium costale* also has a narrower distribution, ranging from Long Island Sound, New York, to Cape Charles, Virginia.

Similar agents have been reported from hatchery-reared pearl oysters, *Pinctada maxima,* (Fig.M.6.1.3a,b) and the rock oyster, *Saccostrea cucullata* (Fig.M.6.1.3c,d), from north Western Australia.

M.6.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

No information or no positive report for this disease in any country for the reporting periods 1999 and 2000 (OIE 1999, OIE 2000b).

M.6.2 **Clinical Aspects**

Haplosporidium nelsoni occurs extracellularly in the connective tissue and digestive gland epithelia. It is often associated with a visible brown-red discolouration of gill and mantle tissues. Sporulation of *H. nelsoni* is prevalent in juvenile oysters (1-2 yrs) but sporadic in adults and occurs exclusively in the epithelial tissues of the digestive tubules. Sporulation of *H. costale* occurs throughout the connective tissues.

Haplosporidum nelsoni infections appear and continue throughout the summer (mid-May to the end of October). Gradual disruption of the digestive gland epithelia is associated with weakening and cumulative mortalities of oysters. A second wave of mortalities may occur in early spring from oysters too weak to survive over-wintering. Holding *in vivo* for up to 2 weeks in 10 ppt salinity seawater at 20°C kills the parasite but not the host. *H. nelsoni* does not cause disease at <15 ppt salinity.

Haplosporidium costale causes a pronounced seasonal mortality between May and June. Sporulation is more synchronous than with MSX infections, causing acute tissue disruption, weakening and death of heavily infected individuals. SSO disease is restricted to salinities of 25-33 ppt and infections appear to be lost at lower salinities.

M.6.3 **Screening Methods**

More detailed methods for screening can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.6.3.1 *Presumptive*

M.6.3.1.1 Gross Observations (Level I)

Slowed growth, gaping and mortalities of *Crassostrea virginica* and *C. gigas* should be considered suspect for Haplosporidiosis. Gross signs are not pathogen specific and require Level II examination, at least for first time observations.

M.6.3.1.2 Cytological Examination and Tissue Imprints (Level II)

As with histology (M.6.3.2.2), juvenile oysters are preferred for cytological or tissue imprint screening for *Haplosporidium nelsoni*. For *H. costale* adult oysters are preferred. Screening during May-June is recommended for both disease agents.

Heart smears or impressions (dabs) can be made onto a clean microscope slide and air-dried. Digestive gland and gill sections can also be used for smears by absorbing excess water from cut surfaces and dabbing the surface onto clean slides. Once dry, the slide is fixed in 70% methanol. Quick and effective staining can be achieved with commercially available blood-staining (cytological) kits, using the manufacturer's instructions. The stained slides are then rinsed (gently) under tap water, allowed to dry, and cover-slipped using a synthetic resin mounting medium.

The presence (especially between March and June in endemic areas) of multinucleate plasmodia measuring 2-15 µm in diameter is indicative of *H. costale* infection (Fig.6.3.1.2a). Plasmodia of *H. nelsoni* are detectable between mid-May and October throughout the tissues and measure 4-30 µm in diameter (Fig.M.6.3.1.2b).

Haemolymph suspensions can also be collected from live oysters, however, this is more time-consuming than heart/tissue imprints and is considered less useful for screening purposes.

M.6.3.1.3 Histopathology (Level II)

For *Haplosporidium nelsoni*, juvenile oysters are preferred for screening. For *H. costale* adult oysters are preferred. Screening during May-June is recommended for both disease agents. The

M.6 Haplosporidiosis (*Haplosporidium costale*, *H. nelsoni*)

(SE McGladdery)

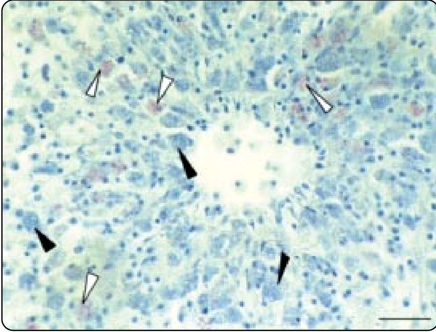

Fig.M.6.3.1.2a. Plasmodia (black arrows) and spores (white arrows) of *Haplosporidium costale*, the cause of SSO disease, throughout the connective tissue of an American oyster (*Crassostrea virginica*). Scale bar 50 µm.

(SE McGladdery)

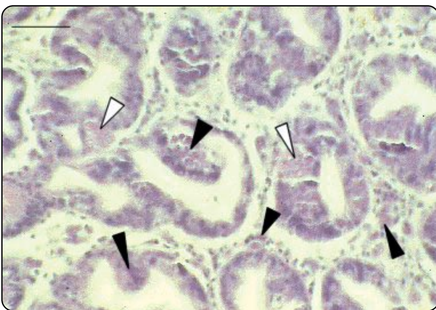

Fig.M.6.3.1.2b. Plasmodia (black arrows) and spores (white arrows) of *Haplosporidium nelsoni*, the cause of MSX disease, throughout the connective tissue and digestive tubules of an American oyster (*Crassostrea virginica*). Scale bar 100 µm.

(SE McGladdery)

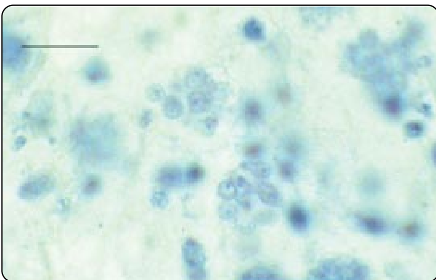

Fig.M.6.4.2.2a. Oil immersion magnification of SSO spores in the connective tissue of an American oyster *Crassostrea virginica*. Scale bar 15 µm.

(SE McGladdery)

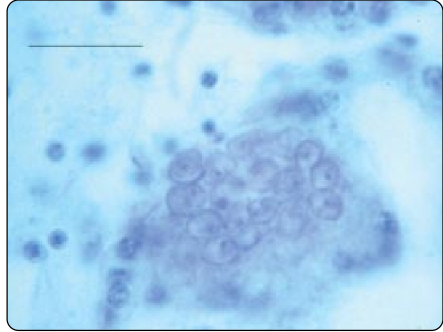

Fig.M.6.4.2.2b. Oil immersion magnification of MSX spores in the digestive tubule epithelium of an American oyster *Crassostrea virginica*. Scale bar 25 µm. (H&E).

techniques used are the same as described for confirmatory diagnosis (M.6.4.2.2).

M.6.4 **Diagnostic Methods**

More detailed methods for diagnostics can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int or selected references.

M.6.4.1 *Presumptive*

M.6.4.1.1 Gross Observations (Level I)

The only presumptive diagnosis would be gross observations of cumulative mortalities of American oysters in early spring and late summer in areas (12-25 ppt salinity) with an established history of MSX epizootics. Such presumptive diagnosis requires confirmation via another diagnostic technique (histology). Likewise, summer mortalities of the same oyster species in waters with a history of SSO disease may be presumed to be due to SSO. Both must be confirmed, however, since infection distributions for both species of *Haplosporidium* may overlap.

M.6.4.2 *Confirmatory*

M.6.4.2.1 Cytological Examination and Tissue Imprints (Level II)

Positive cytological or tissue imprints (M.6.4.1.1) can be considered confirmatory where collected from susceptible oyster species and areas with a historic record of the presence of *Haplosporidium* spp. infections.

M.6.4.2.2 Histopathology (Level II)

Positive histological sections can be considered confirmatory where collected from susceptible oyster species and areas with a historic record of the presence of *Haplosporidium* spp. infections.

It is recommended that at least two dorso-ventral sections through each oyster be examined using oil immersion for screening purposes. Sections from oysters >2 yrs (or >30 mm shell height) should be fixed immediately in a fast fixative such as 1G4F. Davidson's or 10% buffered formalin may be used for smaller or whole oysters (see M.1.3.3.3) but these fixatives are not optimal for subsequent confirmatory Electron Microscopy (EM) diagnosis (M.6.4.2.3), or species identification, if required. Several standard stains (*e.g.*, haemotoxylin-eosin) enable detection of *Haplosprodium* spp..

Haplosporidium spp. infections are usually systemic and characterised by massive infiltration by hyalinocyte haemocytes (agranular haemocytes with a low cytoplasm: nucleoplasm ratio). The sporoplasm of spores of *H. costale* which are smaller than those of MSX and often masked by the intense haemocyte infiltration response, can be differentially stained using a modified Ziehl-Nielsen stain. Sporocyts of *H. costale* occur in the connective tissues (Fig.M.6.3.1.2a), measure approximately 10-25 µm in diameter and contain oval, operculate, spores approximately 3 µm in size (Fig.M.6.4.2.2a). Sporocysts of *H. nelsoni* occur in the digestive tubule epithelia and measure 20-50 µm in diameter. The operculate spores of MSX measure 4-6 x 5-8 µm (Fig.M.6.4.2.2b). In *C. gigas* spores may also occur in other tissues. Older foci of infection in both oyster species may be surrounded by haemocytes and necrotic tissue debris. A similar infectious agent occurs in the pearl oyster *Pinctada maxima* in north Western Australia (Fig.M.6.1.3a,b). The spore size of this *Haplosporidium* resembles *H. nelsoni* but differs from MSX infections in both *C. virginica* and *C. gigas* by being found exclusively in the connective tissue.

The plasmodial stages of both *H. costale* and *H. nelsoni* are as described under M.6.3.1.2.

M.6.4.2.3 Transmission Electron Microscopy (TEM) (Level III)

TEM is required for confirmation of species-specific ultrastructure of the spores - especially in areas enzootic for both disease agents. Tissues are fixed in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater. Oyster tissues can also be fixed in 1G4F for 12-24 hrs. Following primary fixation, rinse tissues in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO_4 = osmic acid - *highly toxic*). Secondary fixation should be complete within 1 hr. The OsO_4 fixative must also be rinsed with buffer/filtered (0.22 µm) seawater prior to dehydration and resin-embedding.
Post-fixed tissues can be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides with 1% toluidine blue solution is one method of selecting the tissue specimens for optimum evidence of *Haplosporidium* plasmodia or spores.

M.6.4.2.4 *In situ* Hybridization (Level III)

DNA-probes for both species of *Haplosporidium* have been produced at the Virginia Institute of Marine Science (VIMS), College of William and Mary, Gloucester, Virginia, USA. These are not yet commercially available, but labelled probes may be obtained for experienced users, or samples may be sent to VIMS[1] for *in situ* hybridization analysis.

M.6.5 **Modes of Transmission**

Neither parasite has been successfully transmitted under laboratory conditions and one (or more) intermediate host(s) is/are suspected.

M.6.6 **Control Measures**

None are known for *Haplosporidium* spp.. Most efforts have concentrated on development of resistant stocks of oysters. These currently show potential for survival in enzootic areas, but are not recommended for use in non-enzootic areas due to their potential as sub-clinical carriers of the pathogen. Some success has also been achieved in preventing infection of hatchery-reared larval and juvenile oysters through filtration and UV radiation of influent water.

[1] Attention Dr. N. Stokes, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062, USA. (E-mail: stokes@vims.edu).

M.6 Haplosporidiosis (*Haplosporidium costale*, *H. nelsoni*)

M.6.7 Selected References

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M.7 MARTEILIOIDOSIS (*MARTEILIOIDES CHUNGMUENSIS*, *M. BRANCHIALIS*)

M.7.1 **Background Information**

M.7.1.1 *Causative Agents*

Marteilioidosis is caused by two species of parasites, belonging to the protistan Phylum Paramyxea. *Marteilioides chungmuenis* is responsible for oocyte infections of Pacific oysters (*Crassostrea gigas*) and *Marteilioides branchialis* infects the gills of *Saccostrea glomerata* (syn. *Crassostrea commercialis, Saccostrea commercialis*).

M.7.1.2 *Host Range*

The Pacific oyster *Crassostrea gigas* is infected by *Marteilioides chungmuensis. Marteilioides branchialis* infects the Sydney rock oyster, *Saccostrea commercialis*.

M.7.1.3 *Geographic Distribution*

Marteilioides chungmuensis infects *C. gigas* in Japan and Korea. *Marteilioides branchialis* is found in Australia (New South Wales).

M.7.2 **Clinical Aspects**

Marteilioides chungmuensis infects the cytoplasm of mature oocytes and significant proportions of the reproductive output of a female oyster can be affected. The infected eggs are released or retained within the follicle, leading to visible distention of the mantle surface (Fig.M.7.2a, b). Prevalences of up to 8.3% have been reported from Korea. *Marteilioides branchialis* causes focal lesions on the gill lamellae and, in conjunction with infections by *Marteilia sydneyi* (M.3), is associated with significant mortalities of Sydney rock oysters being cultured in trays during the autumn.

M.7.3 **Screening Methods**

M.7.3.1 *Presumptive*

M.7.3.1.1 Gross Observations (Level I)

Marteilioides branchialis causes focal patches (1-2 mm in diameter) of discolouration and swelling on the gill lamellae. The presence of such lesions in Sydney rock oysters in the Austral autumn should be treated as presumptive Marteilioidosis.

M.7.3.2 *Confirmatory*

M.7.3.2.1 Histopathology (Level II)

The techniques used are the same as described for confirmatory disease diagnosis (M.7.4.2.1). Presence of histological inclusions, as described under M.7.4.2.1, can be considered confirmatory for *Marteilioides* spp., during screening.

(MS Park and DL Choi)

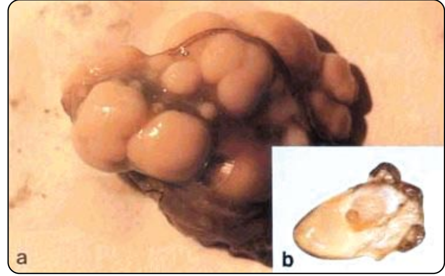

Fig.M.7.2a,b. a. Gross deformation of mantle tissues of Pacific oyster (*Crassostrea gigas*) from Korea, due to infection by the protistan parasite *Marteiloides chungmuensis* causing retention of the infected ova within the ovary and gonoducts; b. (insert) normal mantle tissues of a Pacific oyster.

(MS Park)

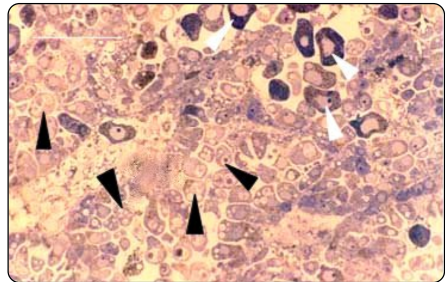

Fig.M.7.4.2.1. Histological section through the ovary of a Pacific oyster (*Crassostrea gigas*) with normal ova (white arrows) and ova severely infected by the protistan parasite *Marteiliodes chungmuensis* (black arrows). Scale bar 100 µm.

M.7.4 **Diagnostic Methods**

M.7.4.1 *Presumptive*

M.7.4.1.1 Gross Observations (Level I)

As for M.7.3.1.1, focal patches (1-2 mm in diameter) of discolouration and swelling on the gill lamellae of Sydney rock oysters in the Austral autumn can be treated as presumptive positives for *M. branchialis*.

M.7.4.1.2 Histopathology (Level II)

For first-time diagnoses a back up tissue specimen fixed for EM is recommended (M.7.4.2.3).

M.7.4.2 *Confirmatory*

M.7.4.2.1 Histopathology (Level II)

Positive histological sections can be considered confirmatory where collected from susceptible oyster species and areas with a historic record of the presence of *Marteilioides* spp. infections.

It is recommended that at least two dorso-ventral sections through each oyster be examined using oil immersion for screening purposes. Sections from oysters >2 yrs (or >30 mm shell height) should be fixed immediately in a fast fixative such as 1G4F. Davidsons or 10% buffered formalin may be used for smaller or whole oysters (see M.1.3.3.3) but these fixatives are not optimal for subsequent confirmatory Electron Microscopy (EM) diagnosis (M.6.4.2.3), or species identification, if required. Several standard stains (*e.g.*, haemotoxylin-eosin) enable detection of *Marteilioides* spp..

Marteilioides chungmuensis is located in the cytoplasm of infected ova (Fig.M.7.4.2.1). Stem (primary) cells contain secondary cells. These may, in turn, contain developing sporonts, giving rise to a single tertiary cell by endogenous budding. Each tertiary cell forms a tricellular spore by internal cleavage.

Marteilioides branchialis causes epithelial hyperplasia and granulocyte infiltration at the site of infection. Uninucleate primary cells contain two to six secondary cells (some may contain up to 12) in the cytoplasm of epithelial cells, connective tissue cells and occasionally the infiltrating haemocytes within the lesion.

M.7.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

TEM is required for confirmation of species-specific ultrastructure of these parasites. Tissues are fixed in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater. Tissues can also be fixed in 1G4F for 12-24 hours. Following primary fixation, rinse in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO_4 = osmic acid - *highly toxic*). Secondary fixation should be complete within 1 hour. The OsO_4 fixative must also be rinsed with buffer/filtered (0.22 microns) seawater prior to dehydration and resin-embedding.

Post-fixed tissues should be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides with 1% toludine blue solution is one method of selecting the tissue specimens for optimum evidence of putative *Marteilioides* spp. Ultrathin sections are then mounted on copper grids (with or without formvar reinforced support) for staining with lead citrate + uranyl acetate or equivalent EM stain.

Marteilioides branchialis is differentiated from the other *Marteilioides* spp. by the presence of two concentric cells (rather than three) within the spore. In addition *M. chungmuensis* in *C. gigas* contains only two to three sporonts per primary/stem cell compared with two-six (or up to 12) for *M. branchialis*. Multivesicular bodies resembling those of *Marteilia* spp. are present in *M. branchialis* stem cells, but absent from those of *M. chungmuensis*.

M.7.5 **Modes of Transmission**

Unknown.

M.7.6 **Control Measures**

None known.

M.7.7 **Selected References**

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M.8 IRIDOVIROSIS (OYSTER VELAR VIRUS DISEASE)

M.8.1 Background Information

M.8.1.1 *Causative Agents*

Oyster Velar Virus Disease (OVVD) (Iridovirosis) is caused by an icosahedral DNA virus with morphological similarities to the Iridoviridae.

M.8.1.2 *Host Range*

Crassostrea gigas (Pacific oyster) larvae are the documented host species, although similar viral agents have been associated with gill disease ("Maladie des Branchies") and haemocyte infections in Portugese oysters (*Crassostrea angulata*) and *C. gigas*.

M.8.1.3 *Geographic Distribution*

Infections have been reported solely from two hatcheries in Washington State, but are believed to have a ubiquitious distribution throughout juvenile *C. gigas* production, with clinical manifestation only under sub-optimal growing conditions.

M.8.2 Clinical Aspects

OVVD causes sloughing of the velar epithelium of larvae >150µm in length, and can cause up to 100% mortality under hatchery conditions. The larvae can not feed, weaken and die.

M.8.3 Screening Methods

M.8.3.1 *Presumptive*

Generally-speaking, since this is an opportunistic infection, only clinical infections will demonstrate detectable infections – as described under M.8.4.

M.8.3.1.1 Wet Mounts (Level I)

Wet mounts of veliger larvae which demonstrate sloughing of ciliated epithelial surfaces can be considered to be suspect for OVVD. As with gross observations, other opportunistic pathogens (bacteria and *Herpes*-like viruses) may be involved, so Level II/III diagnostics are required.

M.8.3.1.2 Histopathology (Level II)

Using the techniques described under M.8.4.2.1, detection the features described in that section can be considered to be presumptively positive for OVVD. Such inclusions require TEM (Level III) (M.8.4.2.2) for confirmatory diagnosis, at least for first time observations.

M.8.3.2 *Confirmatory*

M.8.3.2.1 Transmission Electron Microscopy (Level III)

As described under M.8.4.2.2.

M.8.4 Diagnostic Methods

M.8.4.1 *Presumptive*

M.8.4.1.1 Gross Observations (Level I)

Slowed growth, cessation of feeding and swimming in larval *Crassostrea gigas* should be considered suspect for OVVD. Gross signs are not pathogen specific and require Level II examination (M.8.4.2), at least for first time observations.

M.8.4.1.2 Wet Mounts (Level I)

As described under M.8.3.1.1. For first-time diagnoses a back up tissue specimen fixed for TEM is recommended (M.8.4.2.2).

M.8.4.1.3 Histopathology (Level II)

As described under M.8.4.2.1.

M.8.4.1.4 Transmission Electron Microscopy (Level III)

As described under M.8.4.2.2.

M.8.4.2 *Confirmatory*

M.8.4.2.1 Histopathology (Level II)

Where larvae have a history of OVVD, detection of inclusions and ciliated epithelial pathology, as described below, can be considered confirmatory for the disease. However, it should be noted that other microbial infections can induce similar histopathology and electron microscopy is the ideal confirmatory technique (M.8.4.2.2).

Larvae must be concentrated by centrifugation or filtration into a pellet prior to embedding. This is best achieved post-fixation in Davidson's, 1G4F or other fixative. Although paraffin embedding is possible, resin embedding is recommended for optimal sectioning. Paraffin permits sectioning down to 3 µm using standard microtome. Resin embedded tissue can be sectioned down to 1 µm thick, but requires specialised microtomes and/or block holders and specialised staining.

M.8 Iridovirosis (Oyster Velar Virus Disease)

Standard stains (*e.g.,* haemotoxylin-eosin) will detect intracytoplasmic inclusion bodies in ciliated velar epithelial cells. Early inclusion bodies are spherical, but become more irregular as the viruses proliferate. Inclusion bodies may also be detected in oesophageal and oral epithelia or, more rarely, in mantle epithelial cells.

M.8.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

TEM is required to visualise the causative viruses *in situ* in gill tissue sections of concentrated 'pellets' of larvae. Fixation in 2-3% glutaraldehyde mixed and buffered for ambient filtered seawater should not exceed 1 hour to reduce artifacts. Tissues can also be fixed in 1G4F for 12-24 hrs. Following primary fixation, rinse in a suitable buffer and post-fix in 1-2% osmium tetroxide (OsO_4 = osmic acid - *highly toxic*). Secondary fixation should be complete within 1 hr. The OsO_4 fixative must also be rinsed with buffer/filtered (0.22 µm) seawater prior to dehydration and resin-embedding.

Post-fixed tissues should be stored in a compatible buffer or embedded post-rinsing in a resin suitable for ultramicrotome sectioning. Screening of 1 micron sections melted onto glass microscope slides with 1% toludine blue solution is one method of selecting the best specimens for ultrathin sectioning. Ultrathin sections are mounted on copper grids (with or without formvar reinforced support) for staining with lead citrate + uranyl acetate or equivalent EM stain.

Icosahedral viral particles (228 +/- 7 *nm* in diameter) with a bi-laminar membrane capsid should be evident to confirm Iridoviral involvement.

M.8.5 **Modes of Transmission**

The disease appeared in March-May at affected hatcheries. Direct transmission between moribund and uninfected larvae is suspected.

M.8.6 **Control Measures**

None known except for reduced stocking densities, improved water exchange and general hatchery sanitation methods (tank and line disinfection, *etc.*).

M.8.7 **Selected References**

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Annex M.AI. OIE Reference Laboratory for Molluscan Diseases

Disease	Expert/Laboratory
Mollusc pathogens	**Dr. F. Berth** IFREMER Laboratoire de Genetique Aquaculture et Pathologie BP 133, 17390 La Tremblade FRANCE Tel: 33(0)5 46.36.98.36 Fax: 33 (0)5 46.36.37.51 E-mail: fberthe@ifremer.fr

Annex M.AII. List of Regional Resource Experts for Molluscan Diseases Asia-Pacific[1]

Disease	Expert
Bonamiosis	**Dr. Brian Jones** Senior Fish Pathologist Fisheries WA Adjunct Professor, Muresk Institute c/o Animal Health Labs 3 Baron-Hay Court South Perth WA 6151 **AUSTRALIA** Tel: 61-8-9368-3649 Fax: 61-8-9474-1881 E-mail: bjones@agric.wa.gov.au Marteilioisis/Microcytosis
	Dr. Robert D. Adlard Queensland Museum PO Box 3300, South Brisbane, Queensland 4101 **AUSTRALIA** Tel: +61 7 38407723 Fax: +61 7 38461226 E-mail: RobertAd@qm.qld.gov.au; http://www.qmuseum.qld.gov.au
Marteiliosis	**Dr. Sarah N. Kleeman** Aquatic Animal Biosecurity Animal Biosecurity GPO Box 858 Canberra ACT 2601 **AUSTRALIA** Tel: +61 2 6272 3024 Fax: +61 2 6272 3399 E-mail: sarah.kleeman@affa.gov.au
Molluscan Diseases	**Professor R.J.G. Lester** Department of Microbiology and Parasitology The University of Queensland, Brisbane **AUSTRALIA** 4072. Tel: +61-7-3365-3305, Fax: +61-7-3365-4620 E-mail: R.Lester@mailbox.uq.edu.au http://www.biosci.uq.edu.au/micro/academic/lesterlester.htm
	Dr. Dong Lim Choi Pathology Division #408-1, Shirang-ri, Kijang-up Kijang-gun, Busan 619-902 **KOREA RO** Tel: +82-51-720-2493 Fax: +82-51-720-2498 E-mail: dlchoi@haema.nfrda.re.kr
	Dr. Mi-Seon Park Pathology Division #408-1, Shirang-ri, Kijang-up Kijang-gun, Busan 619-902 **KOREA RO** 619-902 Tel: +82-51-720-2493 Fax: +82-51-720-2498 E-mail: parkms@nfrdi.re.kr

[1] The experts included in this list has previously been consulted and agreed to provide valuable information and health advise concerning their particular expertise.

Annex M.AII. List of Regional Resource Experts for Molluscan Diseases Asia-Pacific

	Dr. Jie Huang Yellow Sea Fisheries Research Institute Chinese Academy of Fishery Sciences 106 Nanjing Road Qingdao, Shandong 266071 **PEOPLE'S REPUBLIC of CHINA** Tel: 86 (532) 582 3062 Fax: 86 (532) 581 1514 E-mail: aqudis@public.qd.sd.cn
	Mr. Arthur de Vera Fish Health Section Bureau of Fisheries and Aquatic Resources Arcadia Building, 860 Quezon Avenue Quezon City, Metro Manila **PHILIPPINES** Fax: (632) 3725055 Tel: (632) 4109988 to 89 E-mail: azdv@edsamail.com.ph
	Dr. Paul Michael Hine Aquatic Animal Diseases National Centre for Disease Investigation MAF Operations, P.O. Box 40-742 Upper Hutt **NEW ZEALAND** Tel: +64-4-526-5600 Fax: +64-4-526-5601 E-mail: hinem@maf.govt.nz
List of Experts Outside Asia-Pacific Region[2]	
Molluscan Diseases	**Dr. Susan Bower** DFO Pacific Biological Station 3190 Hammond Bay Road Nanaimo, British Columbia V9R 5K6 **CANADA** Tel: 250-756-7077 Fax: 250-756-7053 E-mail: bowers@dfo-mpo.gc.ca
	Dr. Sharon E. McGladdery Oceans and Aquaculture Science 200 Kent Street (8W160) Ottawa, Ontario, K1A 0E6 **CANADA** Tel: 613-991-6855 Fax: 613-954-0807 E-mail: mcgladderys@dfo-mpo.gc.ca

[2] These experts outside the Asia-Pacific region has supported the regional programme on aquatic animal health and agreed to assist further in providing valuable information and health advise on molluscan diseases.

Annex M.AIII. List of Useful Diagnostic Manuals/ Guides/Keys to Molluscan Diseases

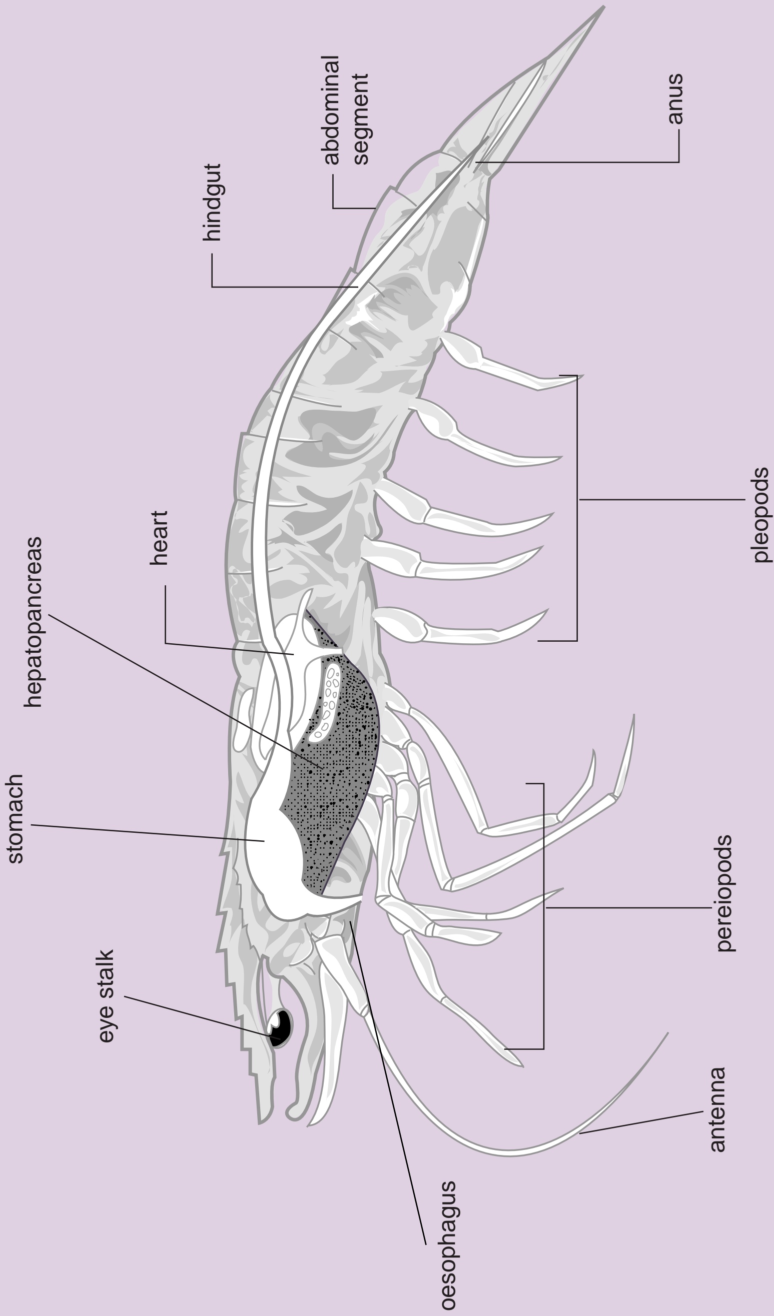
- **Australian Aquatic Animal Disease – Identification Field Guide (1999) by Alistair Herfort and Grant Rawlin**
  Information: AFFA Shopfront – Agriculture, Fisheries and Forestry – Australia
  GPO Box 858, Canberra, ACT 2601
  Tel: (02) 6272 5550 or free call: 1800 020 157
  Fax: (02) 6272 5771
  E-mail: shopfront@affa.gov.au

- **Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish by Bower, SE McGladdery and IM Price (1994)**
  Information: Dr. Susan Bower
  DFO Pacific Biological Station
  3190 Hammond Bay Road
  Nanaimo, British Columbia
  V9R 5K6
  CANADA
  Tel: 250-756-7077
  Fax: 250-756-7053
  E-mail: bowers@dfo-mpo.gc.ca

- **Mollusc Diseases: Guide for the Shellfish Farmer. 1990. by R.A. Elston. Washington Sea Grant Program, University of Washington Press, Seattle. 73 pp**.

- **A Manual of the Parasites, Pests and Diseases of Canadian Atlantic Bivalves**. **1993. by SE McGladdery, RE Drinnan and MF Stephenson.**
  Information: Dr. Sharon McGladdery
  Oceans and Aquaculture Science
  200 Kent Street (8W160)
  Ottawa, Ontario, K1A 0E6
  Tel: 613-991-6855
  Fax: 613-954-0807
  E-mail: mcgladderys@dfo-mpo.gc.ca

Internal and External Anatomy of a Penaeid Shrimp



Internal and external anatomy of a penaeid shrimp.

SECTION 4 - CRUSTACEAN DISEASES

Internal and External Anatomy of a Penaeid Shrimp		154
SECTION 4 - CRUSTACEAN DISEASES		
C.1	**GENERAL TECHNIQUES**	157
C.1.1	**Gross Observations**	157
C.1.1.1	*Behaviour*	157
C.1.1.1.1	*General*	157
C.1.1.1.2	*Mortalities*	157
C.1.1.1.3	*Feeding*	158
C.1.1.2	**Surface Observations**	158
C.1.1.2.1	*Colonisation and Erosion*	158
C.1.1.2.2	*Cuticle Softening, Spots and Damage*	158
C.1.1.2.3	*Colour*	158
C.1.1.2.4	*Environmental Observations*	160
C.1.1.3	*Soft-Tissue Surfaces*	160
C.1.2	**Environmental Parameters**	160
C.1.3	**General Procedures**	160
C.1.3.1	*Pre-collection Preparation*	160
C.1.3.2	*Background Information*	162
C.1.3.3	*Sample Collection for Health Surveillance*	162
C.1.3.4	*Sample Collection for Disease Diagnosis*	162
C.1.3.5	*Live Specimen Collection for Shipping*	162
C.1.3.6	*Preservation of Tissue Samples*	164
C.1.3.7	*Shipping Preserved Samples*	165
C.1.4	**Record-Keeping**	165
C.1.4.1	*Gross Observations*	165
C.1.4.2	*Environmental Observations*	165
C.1.4.3	*Stocking Records*	166
C.1.5	**References**	166
	VIRAL DISEASES OF SHRIMP	
C.2	**Yellowhead Disease (YHD)**	167
C.3	**Infectious Hepatopancreas and Haematopoietic Necrosis (IHHN)**	173
C.4	**White Spot Disease (WSD)**	178
C.4a	**Bacterial White Spot Syndrome (BWSS)**	183
C.5	**Baculoviral Midgut Gland Necrosis (BMN)**	186
C.6	**Gill-Associated Virus (GAV)**	189
C.7	**Spawner Mortality Syndrome ("Midcrop mortality syndrome")**	192
C.8	**Taura Syndrome (TS)**	194
C.9	**Nuclear Polyhedrosis Baculovirosis (NPD)**	201
	BACTERIAL DISEASE OF SHRIMP	
C.10	**Necrotising Hepatopancreatitis (NH)**	207
	FUNGAL DISEASE OF CRAYFISH	
C.11	**Crayfish Plague**	211
	ANNEXES	
C.AI	**OIE Reference Laboratories for Crustacean Diseases**	215

Section 4 - Crustacean Diseases

C.AII	**List of Regional Resource Experts for Crustacean Diseases in the Asia-Pacific**	216
C.AIII	**List of Useful Manuals/Guide to Crustacean Diseases in Asia-Pacific**	219

List of National Coordinators(NCs) 221
Members of Regional Working Group (RWG) and Technical Support Services (TSS) 225
List of Figures 230

C.1 GENERAL TECHNIQUES

General crustacean health advice and other valuable information are available from the OIE Reference Laboratories, Regional Resource Experts in the Asia-Pacific, FAO and NACA. A list is provided in Annexes F.AI and AII, and up-to-date contact information may be obtained from the NACA Secretariat in Bangkok (E-mail:naca@enaca.org) . Other useful guides to diagnostic procedures which provide valuable references for crustacean diseases are listed in Annex F.AIII.

C.1.1 **Gross Observations**

Gross observations of clinical signs in shrimp can be easily made at the farm or pond side using little, if any, equipment. Although, in most cases, such observations are insufficient for a definite diagnosis, such information is essential for preliminary compilation of a strong "case description" (or case history). Accurate and detailed gross observations also help with initiation of an action plan which can *effectively* reduce losses or spread of the disease, *e.g.*, destruction or isolation of affected stocks, treatments or alterations to husbandry practices (*i.e.*, feeding regimes, stocking densities, pond fertilisation, *etc.*). These can all be started while waiting for more conclusive diagnostic results.

C.1.1.1 *Behaviour* (Level 1)

C.1.1.1.1 General

Abnormal shrimp behaviour is often the first sign of a stress or disease problem. Farmers or farm workers, through daily contact with their stocks, rapidly develop a subconscious sense of when "something is wrong". This may be subtle changes in feeding behaviour, swimming movement or unusual aggregations. Even predator activity can provide clues to more "hidden" changes such as when fish- or shrimp-eating birds congregate round affected ponds. Record-keeping (see C.1.4) can provide valuable additional evidence that reinforces such observations and can indicate earlier dates when problems started to appear. It is important that farmers and workers on the farm, as well as field support staff, get to know the "normal" (healthy) behaviour of their stocks. Since some species and growing environments may demonstrate or evoke subtle differences in behaviour, these should be taken into account, especially if changing or adding species, or when information gathered from a different growing environment is used. Where any change from normal behaviour affects more than small numbers of random individuals, this should be considered cause for concern and warrants investigation.

Some clues to look out for in shrimp stocks include:

- unusual activity during the daytime - shrimps tend to be more active at night and stick to deeper water during the day
- swimming at or near pond surface or edges - often associated with lethargy (shrimp swimming near the surface may attract predatory birds)
- increased feed consumption followed by going off-feed
- reduction or cessation of feeding
- abnormal feed conversion ratios, length/ weight ratios
- general weakening - lethargy (*note: lethargy is also characteristic in crustaceans when the water temperature or dissolved oxygen levels are low, so these possibilities should be eliminated as potential causes before disease investigations are started*)

C.1.1.1.2 Mortalities

Mortalities that reach levels of concern to a producer should be examined for any patterns in losses, such as:

- **relatively uniform** mortalities throughout a system should be examined immediately and environmental factors determined (ideally with pre-mortality records - see C.1.4)
- **apparently random**, or sporadic mortalities may indicate a within-system or stock problems. If the following conditions exist - (a) no history of stock-related mortalities, (b) all stock originate from the same source, and (c) there have been no changes to the rearing system prior to mortality problems - samples of affected and unaffected shrimp should be submitted for laboratory examination (Level II or III), as appropriate, and supported by gross observations and stock history (see C.1.4)
- **mortalities that spread** suggest an infectious cause and should be sampled immediately. Affected shrimp should be kept as far away as possible from unaffected shrimp until the cause of the mortalities can be established.

C.1 General Techniques

C.1.1.1.3 Feeding

Absence of feeding behaviour and lack of feed in the gut are good indicators of potential problems. Daily gut content checks can be made on shrimp caught in feeding trays or bowls (where used) or, less frequently, from samples taken to determine growth. Ideally examination of feeding behaviour should be made every 1-2 weeks, even in extensive farming systems. Feeding behaviour is most easily checked by placing feed in a tray or bowl (Fig.C.1.1.1.3a) and seeing how quickly the shrimp respond, ideally after the shrimp have not been fed for at least a few hours. It is important that the feed used is attractive to the shrimp as poorly formulated, old or badly stored feeds may not be attractive to the shrimp. Gut contents can be checked by holding the shrimp against a light to show the gut in the tail segments (Fig.C.1.1.1.3b). If these are empty, especially just after providing feed, it may indicate either of the following conditions: i) underfeeding, or ii) onset of cessation of feeding (anorexia).

Where possible, feed records (see C.1.4) should be maintained to determine normal feed consumption patterns (*i.e.*, feeding activity by healthy shrimp), which can be compared with "suspect" feeding activity. In many cases of chronic loss, daily feed consumption patterns may remain stable or oscillate over periods of several weeks. These can be detected by making a graph of daily feed consumption or by comparing daily feed consumption in the record book over an extended period (*e.g.* 3-4 weeks).

C.1.1.2 *Surface Observations* (Level 1)

C.1.1.2.1 Colonisation and Erosion

Colonisation of the shell (cuticle) and gills of a crustacean is an on-going process that is usually controlled by grooming. The presence of numerous surface organisms (*e.g.* "parasites" - which damage their host; or "commensals" - that do not adversely impact their host) suggests sub-optimal holding conditions or a possible disease problem. Apparent wearing away (erosion) of the cuticle or appendages (legs, tail, antennae, rostrum) (Fig.C.1.1.2.1a), or loss of appendages, with or without blackening (melanization) are also highly indicative of a disease problem. Breakage of the antennae is an early warning sign. In healthy penaeid shrimp, these should extend approximately 1/3 past the length of the body (when bent back along the body line). Likewise, erosion or swelling of the tail (uropods and telson), with or without blackening, is an early sign of disease (Fig.C.1.1.2.1b).

C.1.1.2.2 Cuticle Softening, Spots and Damage

Softening of the shell (Fig.C.1.1.2.2a and Fig.C.1.1.2.2b), other than during a moult, may also indicate the presence of infection. Damage or wounds to the shell provide an opportunity for opportunistic infections (mainly bacterial and fungal) to invade the soft-tissues and proliferate, which can seriously impact the health of the shrimp.

Certain diseases, such as White Spot Disease, directly affect the appearance of the shell, however, few changes are specific to a particular infection. In the case of white spots on the cuticle, for example, recent work (Wang *et al.* 2000) has shown that a bacteria can produce signs similar to those produced by White Spot Disease (see C.4) and Bacterial White Spot Syndrome (see C.4a).

C.1.1.2.3 Colour

Shrimp colour is another good indicator of health problems. Many crustaceans become more reddish in color when infected by a wide range of organisms, or when exposed to toxic conditions (Fig.C.1.1.2.3a), especially those that affect the hepatopancreas. This is thought to be due to the release of yellow-orange (carotenoid) pigments that are normally stored in the hepatopancreas. This red colour is not specific for any single condition (or groups of infections), however, so further diagnosis is needed.

Yellowish coloration of the cephalothorax is associated with yellowhead disease (see C.2) and overall reddening can be associated with gill associated virus infections (see C.6), white spot disease or bacteria, as described above, or bacterial septicemia (see C.10). In some cases, the colour changes are restricted to extremities, such as the tail fan or appendages (Fig.C.1.1.2.3b), and these should be examined closely.

It should be noted that some shrimp broodstock, particularly those from deeper waters, can be red in colour (thought to be due to a carotenoid-rich diet). This does not appear to be related to health and its normality can be established through familiarisation with the species being grown. Under certain conditions, some crustaceans may turn a distinct blue

C.1 General Techniques

(P Chanratchakool)

Fig.C.1.1.1.3a. Behaviour observation of shrimp PL in a bowl.

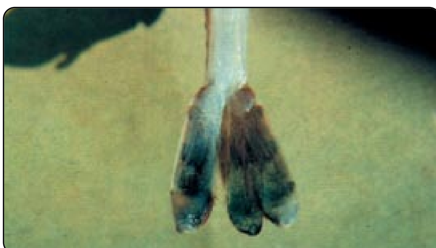
(P Chanratchakool)

Fig.C.1.1.1.3b. Light coloured shrimp with full guts from a pond with healthy phytoplankton.

(P Chanratchakool)

Fig.C.1.1.2.1a. Black discoloration of damaged appendages.

(P Chanratchakool)



(P Chanratchakool/MG Bondad-Reantaso)

Fig.C.1.1.2.2a,b. Shrimp with persistent soft shell.

(P Chanratchakool)

Fig.C.1.1.2.3a. Abnormal blue and red discoloration.

(P Chanratchakool)

Fig.C.1.1.2.3b. Red discoloration of swollen appendage.

Fig.C.1.1.2.1b. Swollen tail due to localized bacterial infection.

colour. This has been shown to be due to low levels of a carotenoid pigment in the hepatopancreas (and other tissues), which may be induced by environmental or toxic conditions. Normal differences in colouration (light to dark) within a species may be due to other environmental variables. For example, *Penaeus monodon* grown in low salinities, are often much paler than *P. monodon* grown in brackish-water or marine conditions. These variations do not appear to be related to general health.

C.1.1.2.4 Environmental Observations

Shrimp with brown gills or soft shells (or a representative sub-sample), should be transferred to a well aerated aquarium with clean sea water at the same salinity as the pond from which they came. They should be observed every 1-2 hrs over 1 day. If the shrimp return to normal activity within a few hours, check environmental parameters in the rearing pond(s).

C.1.1.3 *Soft-Tissue Surfaces* (Level 1)

A readily observable change to soft tissues is fouling of the gill area (Fig. C.1.1.3a), sometimes accompanied by brown discoloration (Fig. C.1.1.3b) (see C.1.1.2.4). This can be due to disease and should trigger action since it reduces the shrimp's ability to take up oxygen and survive.

Removal of the shell in the head region of shrimp allows gross examination of the organs in this region, particularly the hepatopancreas (Fig.C.1.1.3c). In some conditions, the hepatopancreas may appear discoloured (*i.e.*, yellowish, pale, red), swollen or shrunken, compared with healthy shrimp. If the hepatopancreas is gently teased out of the shell, the mid-gut will become visible and permit direct examination of colour (dark - feeding; light/white/yellow - mucoid, empty or not feeding - see C.1.1.1.3). This information is useful for determining the health of the shrimp and if infectious disease agents are present.

C.1.2 **Environmental Parameters** (Level 1)

Environmental conditions can have a significant effect on crustacean health, both directly (within the ranges of physiological tolerances) and indirectly (enhancing susceptibility to infections or their expression). Examples include changes to dissolved oxygen levels and/or pH which may promote clinical expression of previously latent yellowhead disease (see C.2) and white spot disease (see C.4) or the effect of salinity on the expression of necrotising hepatopancreatitis (see C.10). This is especially important for species grown under conditions that bear little resemblance to the wild situation. Water temperature, salinity, turbidity, fouling and plankton blooms (Fig. C.1.2 a,b,c and d) are all important factors. Rapid changes in conditions, rather than gradual changes, are particularly important as potential triggers for disease. Therefore, the farm manager and workers, should attempt to keep pond rearing conditions within the optimum range for the species *and* as constant as possible within that range. High stocking rates are common in aquaculture but predispose individuals to stress so that even minor changes in environmental conditions may precipitate disease. In addition, many small changes do not, on their own, affect shrimp health. However, when several of these small changes occur simultaneously, results can be far more severe.

C.1.3 **General Procedures**

C.1.3.1 *Pre-collection Preparation* (Level I)

The diagnostic laboratory which will be receiving the sample should be consulted to ascertain the best method of transportation (*e.g.*, on ice, preserved in fixative, whole or tissue samples). The laboratory will also indicate if both clinically affected, as well as apparently healthy individuals, are required for comparative purposes. As noted under C.1.3.3 and C.1.3.4, screening and disease diagnosis often have different sample-size requirements.

The laboratory should be informed of exactly what is going to be sent (*i.e.*, numbers, size-classes or tissues) and the intended date of collection and delivery, as far in advance as possible. For screening healthy animals, sample sizes are usually larger so more lead time is required by the laboratory. Screening can be also be planned ahead of time, based on predicted dates of shipping post-larvae (PL) or broodstock, which means the shipper has more time to notify the laboratory well in advance. In cases of disease outbreaks and significant mortalities, there may be less opportunity for advance warning for the laboratory. *However*, the laboratory should still be contacted *prior* to shipment or hand-delivery of any diseased samples (for the reasons given under C.1.3.4). Some samples may require secured packaging or collection by designated personnel, if there are national or international certification

(P Chanratchakool)

Fig.C.1.1.3a. Severe fouling on the gills.

(P Chanratchakool)

Fig.C.1.1.3b. Brown discolouration of the gills.

(P Chanratchakool)

Fig.C.1.1.3c. Shrimp on left side with small hepatopancreas.

(V Alday de Graindorge and TW Flegel)

(P Chanratchakool)

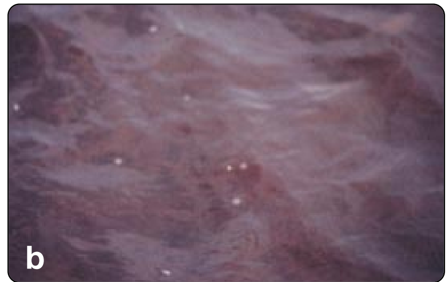


Fig.C.1.2a, b, c. Examples of different kinds of plankton blooms (a- yellow/green coloured bloom; b- brown coloured bloom; c- blue-green coloured bloom.

(P Chanratchakool)

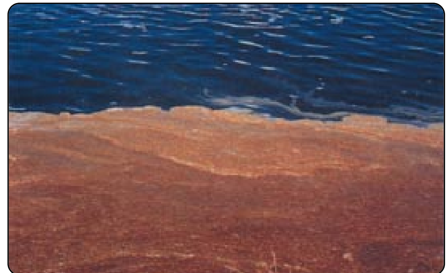


Fig.C.1.2d. Dead phytoplankton.

Fig. C.1.3.6. Points of injection of fixative.

requirements or risk of disease spread via transport of the sample to an area non-endemic for a suspected disease.
Pre-collection discussions with the diagnostic laboratory can significantly speed up processing and diagnosis of a sample (days to weeks) since it allows preparation of the required diagnostic materials in advance of arrival of the sample(s) and ensures that emergency samples are scheduled in for rapid diagnosis.

C.1.3.2 *Background Information* (Level 1)

All samples submitted for diagnosis should include as much supporting information as possible including:

- Gross observations and a history of environmental parameters (as described under C.1.1 and C.1.2)
- Approximate prevalence and pattern of mortality (acute or chronic/sporadic cumulative losses)
- History and origin of affected population
- If the stock is not local, their origin(s) and date(s) of transfer should be included
- Details of feed, consumption rates and any chemical treatments used

The above information provides valuable background details which can help focus attention on possible handling stress, changes in environment or infectious agents as the primary cause of any health problems.

C.1.3.3 *Sample Collection for Health Surveillance*

The most important factors associated with collection of specimens for surveillance are:

- sample numbers that are high enough to ensure adequate pathogen detection (see C.1.3.1 and Table C.1.3.3). Check the number of specimens required by the laboratory before collecting the sample(s) and ensure that each specimen is intact. Larger numbers are generally needed for screening purposes, compared to numbers required for disease diagnosis;
- susceptible species are sampled;
- samples include age- or size-groups that are most likely to manifest detectable infections. Such information is given under the specific disease sections; and
- samples are collected during the season when infections are known to occur. Such information is also given under the specific disease sections.

As mentioned under C.1.3.1, check whether or not designated personnel are required to do the collection, or if secured packaging is necessary, or whether samples are being collected to meet national or international certification requirements.

C.1.3.4 *Sample Collection for Disease Diagnosis*

All samples submitted for disease diagnosis should include as much supporting information as possible, as described under C.1.3.2, with particular emphasis on:

- rates and levels of mortality compared with "normal" levels for the time of year;
- patterns of mortality (random/sporadic, localised, spreading, widespread);
- history and origin(s) of the affected population(s); and
- details of feed used, consumption rates and any chemical treatments.

As in C.1.3.2, the above information will help clarify whether or not an infectious agent is involved and will enable to focus the investigative procedures required for an accurate diagnosis. This information is also *critical* for laboratories outside the region or areas where the suspected disease is endemic. Under such circumstances, the laboratory may have to prepare for strict containment and sterile disposal of all specimen shipping materials and waste products, in order to prevent escape from the laboratory.

Wherever possible**,** check the number of specimens required by the laboratory for diagnostic examination *before* collecting the sample(s). Also check with the laboratory to see whether or not they require specimens showing clinical signs of disease only, or sub-samples of both apparently healthy individuals *and* clinically affected specimens from the same pond/site. The latter option is usually used where a disease-outbreak or other problem is detected for the first time. Comparative samples can help pinpoint abnormalities in the diseased specimens.

C.1.3.5 *Live Specimen Collection for Shipping* (Level 1)

Once the required sample size is determined, the crustaceans should be collected from the water. This should take place as close to shipping as possible to reduce possible mortalities during transportation (especially important for moribund or diseased samples). Wherever pos-

	Prevalence (%)						
Population Size	0.5	1.0	**2.0**	3.0	4.0	**5.0**	10.0
50	46	46	46	37	37	29	20
100	93	93	76	61	50	43	23
250	192	156	110	75	62	49	25
500	314	223	127	88	67	54	26
1000	448	256	136	92	69	55	27
2500	512	279	142	95	71	56	27
5000	562	288	145	96	71	57	27
10000	579	292	146	96	72	29	27
100000	594	296	147	97	72	57	27
1000000	596	297	147	97	72	57	27
>1000000	600	300	**150**	100	75	**60**	30

Table C.1.3.3[1]. Sample sizes needed to detect at least one infected host in a population of a given size, at a given prevalence of infection. Assumptions of 2% and 5% prevalences are most commonly used for surveillance of presumed exotic pathogens, with a 95% confidence limit.

sible, ensure that each specimen is intact.

As noted under C.1.3.1, inform the laboratory of the estimated time of arrival of the sample so they can have the materials required to process prepared before the samples arrive. This shortens the time between removal from the pond and preparation of the specimens for examination.

The crustaceans should be packed in seawater in double plastic bags with the airspace in the bag filled with oxygen. The bags should be sealed tightly with rubber bands or rubber rings and packed inside a foam box. A small amount of ice may be added to keep the water cool, especially if a long transport time is expected. This box is then taped securely and may be packaged inside a cardboard carton. Check with the diagnostic laboratory about packing requirements. Some laboratories have specific packaging requirements for diseased organisms. Samples submitted for certification purposes may have additional shipping and collection requirements (see C.1.3.3).

Label containers clearly:

"LIVE SPECIMENS, STORE AT ___ to ___°C, DO NOT FREEZE"
(insert temperature tolerance range of shrimp being shipped)

If being shipped by air also indicate:

"HOLD AT AIRPORT AND CALL FOR PICK-UP"

- Clearly indicate the name and telephone number of the contact person responsible for picking up the package at the airport or receiving it at the laboratory.
- Where possible, ship ***early in the week*** to avoid arrival during the weekend which may lead to loss through improper storage of samples.
- Inform the contact person as soon as the shipment has been sent and, where appropriate, give them the name of the carrier, the flight number, the waybill number and the estimated time of arrival.

(Note: Some airlines have restrictions on shipping of seawater or preserved samples. It is a good idea to check with local airlines if they do have any special requirements)

[1] Ossiander, F.J. and G. Wedermeyer. 1973. Journal Fisheries Research Board of Canada 30:1383-1384.

C.1.3.6 *Preservation of Tissue Samples* (Level 2)

In some cases, such as locations remote from a diagnostic laboratory or where transport connections are slow, it may not be possible to provide a live shrimp sample. Since freezing is usually inadequate for most diagnostic techniques (histology, bacteriology, mycology, *etc.*), specimens should be fixed (chemical preservation to prevent tissue breakdown and decay) on site. This makes the sample suitable for subsequent histological examination, *in situ* hybridization, PCR or electron microscopy, but will prevent routine bacteriology, mycology, virology or other techniques requiring live micro-organisms. ***Diagnostic needs should therefore be discussed with the laboratory prior to collecting the sample.***

The best general fixative for penaeid shrimp is **Davidson's fixative**.

330 ml 95% ethanol
220 ml 100% formalin (37% w/v formaldehyde in aqueous solution)
115 ml glacial acetic acid
335 ml distilled water.
Mix and store at room temperature.

(It should be noted, however, that formalin residues can interfere with the PCR process. Samples for PCR analysis should be fixed in 70% ethanol.)

For any preservation procedure, it is essential to remember that the main digestive organ of the shrimp (the hepatopancreas) is very important for disease diagnosis, but undergoes rapid autolysis (tissue digestion by digestive juices released from the dying hepatopancreatic cells) *immediately* after death. This means that the pre-death structure of the hepatopancreas is rapidly lost (turns to mush). Delays of even a few seconds in fixative penetration into this organ can result in the whole specimen being useless for diagnosis, thus, specimens must be immersed or injected with fixative *while still alive*. Dead shrimp, even when preserved on ice (or frozen) are of no use for subsequent fixation. In tropical areas, it is best to use cold fixative that has been stored in the freezer or kept on ice, as this helps arrest autolysis and secondary microbial proliferation, as the tissues are preserved.

Larvae and early post larvae (PL) should be immersed directly in a *minimum* of 10 volumes of fixative to one volume of shrimp tissue. This 10:1 ratio is critical for effective preservation. Attempts to cut costs by using lower ratios of fixative to tissue can result in inadequate preservation of tissues for processing.

For PL that are more than approximately 20 mm in length, use a fine needle to make a small, shallow incision that breaks and slightly lifts the cuticle in the midline of the back, at the cuticular junction between the cephalothorax and first abdominal segment. This allows the fixative to penetrate the hepatopancreas quickly.

For larger PL's, juveniles and adults, the fixative should be injected directly into the shrimp, as follows:

- Place the shrimp briefly in ice water to sedate them
- Using surgical rubber gloves and protective eyeglasses, immediately inject the fixative (approximately 10% of the shrimp's body weight) at the following sites (Fig. C.1.3.6):
  - hepatopancreas
  - region anterior to the hepatopancreas
  - anterior abdominal region, and
  - posterior abdominal region.

Be careful to hold the shrimp so the angle of injection is pointed away from your body, since fixative can sometimes spurt back out of an injection site when the needle is removed and may injure the eyes. It is also best to brace the injection hand against the forearm of the hand holding the shrimp, to avoid over penetration of the needle into that hand. The hepatopancreas should receive a larger proportion of the injected fixative than the abdominal region. In larger shrimp it is better to inject the hepatopancreas at several points. All signs of life should cease and the colour should change at the injection sites.

Immediately following injection, slit the cuticle with dissecting scissors along the side of the body from the sixth abdominal segment to the cuticle overlying the "head region" (cephalothorax). From there, angle the cut forward and upward until it reaches the base of the rostrum. Avoid cutting too deeply into the underlying tissue. Shrimp over 12 g should be transversely dissected, at least once, posterior of the abdomen/cephalothorax junction and again mid-abdominally. The tissues should then be immersed in a 10:1 volume ratio of fixative to tissue, at room temperature. The fixative can be changed after 24-72 hr to 70% ethanol, for long-term storage.

C.1.3.7 *Shipping Preserved Samples* (Level 1)

For shipping, remove specimens from ethanol storage, wrap in paper towel saturated with 50% ethanol and place in a sealed plastic bag. There should be no free liquid in the bag. Seal and place within a second sealed bag. In most countries, small numbers of such specimens can be sent to diagnostic laboratories by air-mail. However, some countries or transport companies (especially air couriers) have strict regulations regarding shipping any chemicals, including fixed samples for diagnostic examination. Check with the post office or carrier **before** collecting the samples to ensure they are processed and packed in an appropriate and acceptable manner. All sample bags should be packed in a durable, leak-proof container.

Label containers clearly with the name and telephone number of the contact person responsible for picking up the package at the airport or receiving it at the laboratory.

If being shipped by air indicate - "HOLD AT AIRPORT AND CALL FOR PICK-UP".

Where possible, ship early in the week to avoid arrival during the weekend which may lead to loss through improper storage of samples. Inform the contact person as soon as the shipment has been sent and, where appropriate, give them the name of the carrier, the flight number, the waybill number and the estimated time of arrival.

C.1.4 **Record-Keeping** (Level 1)

Record-keeping is essential for effective disease management. For crustaceans, many of the factors that should be recorded on a regular basis are outlined in sections C.1.1 and C.1.2. It is critical to establish and record normal behaviour and appearance to compare with observations during disease events.

C.1.4.1 *Gross Observations* (Level 1)

These could be included in routine logs of crustacean growth which, ideally, would be monitored on a regular basis either by sub-sampling from tanks or ponds, or by "best-guess" estimates from surface observations.

For hatchery operations, the minimum essential information which should be recorded/logged include:

- feeding activity and feed rates
- growth/larval staging
- mortalities
- larval condition

These observations should be recorded on a daily basis for all stages, and include date, time, tank, broodstock (where there are more than one) and food-source (*e.g.*, brine shrimp culture batch or other food-source). Dates and times for tank and water changes should also be noted, along with dates and times for pipe flushing and/or disinfection. Ideally, these logs should be checked regularly by the person responsible for the site/animals.

Where possible, hatcheries should invest in a microscope and conduct daily microscopic examinations of the larvae. This will allow them to quickly identify problems developing with their stocks, often before they become evident in the majority of the population.

For pond sites, the minimum essential observations which need to be recorded/logged include:

- growth
- feed consumption
- fouling
- mortality

These should be recorded with date, site location and any action taken (e.g., sample collection for laboratory examination). It is important to understand that *rates* of change for these parameters are essential for assessing the cause of any disease outbreak. This means levels have to be logged on a regular and consistent basis in order to detect patterns over time. Ideally, these logs should be checked regularly by the person responsible for the site/animals.

C.1.4.2 *Environmental Observations* (Level 1)

This is most applicable to open ponds. The minimum essential data that should be recorded are:

- temperature
- salinity
- pH
- turbidity (qualitative evaluation or secchi disc)
- algal bloom(s)
- human activity (treatments, sorting, pond changes, etc.)
- predator activity

As with C.1.4.1, types and rates of changes in these parameters *prior* to any disease outbreaks are extremely important in assessing the cause of the outbreak. Although helpful, data recorded on the day of specimen collection are much less useful than continuous records. Thus, the importance of keeping careful, regular and continuous records, regardless of the "expected" results, cannot be overstressed.

Frequency of record-keeping will vary with site and, possibly, season. For example, more frequent monitoring may be required during unstable weather, compared to seasons with extended, stable, conditions.

Human and predator activity should be logged on an "as it happens" basis.

C.1.4.3 *Stocking Records* (Level 1)

All movements of crustaceans into and out of a hatchery and pond/site should be recorded. These should include:

- the exact source of the broodstock or larvae and any health certification history (*e.g.*, results of any tests carried out prior to/on arrival)
- condition on arrival
- date, time and person responsible for receiving delivery of the stock
- date, time and destination of stock shipped out of the hatchery

In addition, all movements of stocks *within* a hatchery, nursery or grow out site should be logged with the date for tracking purposes if a disease situation arises.
Where possible, animals from different sources should not be mixed. If mixing is unavoidable, keep strict records of when mixing occurred.

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VIRAL DISEASES OF SHRIMP
C.2 YELLOWHEAD DISEASE (YHD)[1]

C.2.1 **Background Information**

C.2.1.1 *Causative Agent*

Yellowhead disease (YHD) is caused by Yellowhead Virus (YHV) (also reported in older literature as Yellowhead Baculovirus - YBV and Yellowhead Disease Baculovirus - YHDBV). It is now known not to be a member of the Baculoviridae. YHV is a single stranded RNA, rod shaped (44 ± 6 X 173 ±13 *nm*), enveloped cytoplasmic virus, likely related to viruses in the Family Coronaviridae. Agarose gel electrophoresis indicates a genome size of approximately 22 Kilobases. Lymphoid organ virus (LOV) and gill associated virus (GAV) (see C.6) of *Penaeus monodon* in Australia are related to the YHV complex viruses, although, of the two, only GAV is known to cause mortality. More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a) and Lightner (1996).

C.2.1.2 *Host Range*

Natural infections occur in *Penaeus monodon*, but experimental infections have been shown in *P. japonicus, P. vannamei, P. setiferus, P. aztecus, P. duorarum* and *P. stylirostris. Penaeus merguiensis*, appear to be resistant to disease (but not necessarily infection). *Palaemon styliferus* has been shown to be a carrier of viable virus. *Euphausia* spp. (krill), *Acetes* spp. and other small shrimp are also reported to carry YHD viruses.

C.2.1.3 *Geographic Distribution*

YHD affects cultivated shrimp in Asia including China PR, India, Philippines and Thailand.
YHD has been reported from cultured shrimp in Texas and one sample has been reported to be positive for YHV by antibody assay (Loh *et al.* 1998).

C.2.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

YHD was reported in Malaysia in June, in the Philippines in January to March and July; in Sri Lanka in January and suspected for the whole year of 1999 in Thailand. For the reporting period for the year 2000, India reported it in October and it was suspected for the whole year in Thailand and Sri Lanka (OIE 1999, OIE 2000b).

C.2.2 **Clinical Aspects**

Gross signs of disease (Fig.C.2.2) and mortality occur within 2 to 4 days following an interval of exceptionally high feeding activity that ends in abrupt cessation of feeding. Mortalities can reach 100% within 3-5 days. Diseased shrimp aggregate at the edges of the ponds or near the surface. The hepatopancreas becomes discoloured which gives the cephalothorax a yellowish appearance, hence the name of the disease. The overall appearance of the shrimp is abnormally pale. Post-larvae (PL) at 20-25 days and older shrimp appear particularly susceptible, while PL<15 appear resistant.

Care must be taken in gross diagnosis as mortalities caused by YHD have been reported in the absence of the classic yellowish appearance of the cephalothorax. Clinical signs are not always present, and their absence does not rule out the possibility of YHD infection. Further confirmatory diagnosis including a minimum of whole, stained gill mounts and haemolymph smears should be made in any cases of rapid unexplained mortality in which YHV involvement cannot be ruled out.

YHD virions are found generally in tissues of ectodermal and mesodemal embryonic origin, including: interstitial tissues of the hepatopancreas, systemic blood cells and developing blood cells in the haematopoietic tissues and fixed phagocytes in the heart, the lymphoid (Oka) organ, gill epithelial and pilar cells, connective and spongioform tissues, sub-cuticular epidermis, striated and cardiac muscles, ovary capsules, nervous tissue, neurosecretory and ganglial cells, stomach, mid-gut and midgut caecal walls. The epithelial cells of hepatopancreatic tubules, midgut and midgut caecae (endodermal origin) are not infected with YHV although underlying muscle and connective tissues are. The Oka organ, gill, heart and subcuticular tissues, including those of the stomach epithelium, contain the highest levels of YHV. Infected cells show nuclear pyknosis and karyorrhexis which are apparently signs of viral triggered apoptosis (Khanobdee *et al.* 2001).

C.2.3 **Screening Methods**

More detailed information on methods for screening YHD can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or at selected references.

[1] Yellowhead disease (YHD) is now classified as an OIE Notifiable Disease (OIE 2000a).

C.2 Yellowhead Disease (YHD)

(TW Flegel)

Fig.C.2.2. Gross sign of yellow head disease (YHD) are displayed by the three *Penaeus monodon* on the left.

(DV Lightner)

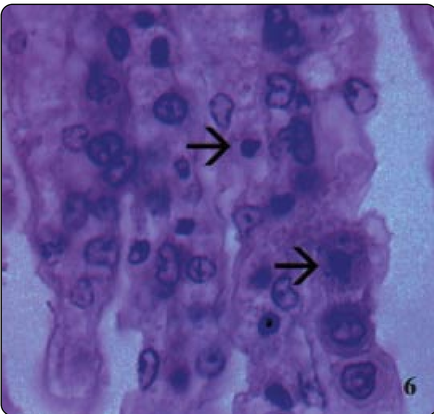
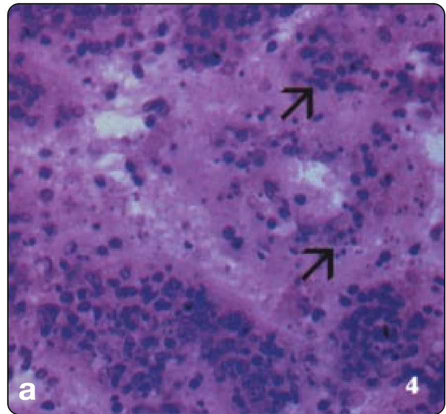


Fig.C.2.3.1.4c. Histological section of the gills from a juvenile *P. monodon* with YHD. A generalized diffuse necrosis of cells in the gill lamellae is shown, and affected cells display pyknotic and karyorrhectic nuclei (arrows). A few large conspicuous, generally spherical cells with basophilic cytoplasm are present in the section. These cells may be immature hemocytes, released prematurely in response to a YHV-induced hemocytopenia. Mayer-Bennett H&E. 1000x magnification.

(DV Lightner)



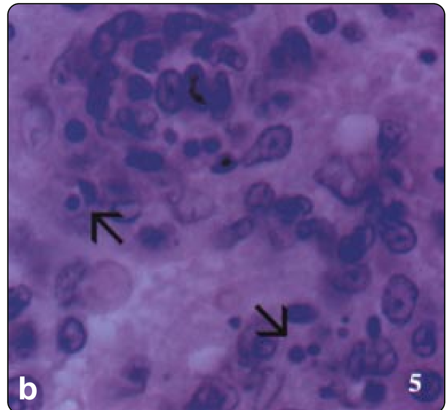


Fig.C.2.3.1.4a,b. Histological section of the lym phoid organ of a juvenile *P. monodon* with severe acute YHD at low and high magnification. A generalized, diffuse necrosis of LO cells is shown. Affected cells display pyknotic and karyorrhectic nuclei. Single or multiple perinuclear inclusion bodies, that range from pale to darkly basophilic, are apparent in some affected cells (arrows). This marked necrosis in acute YHD distinguishes YHD from infections due to Taura syndrome virus,which produces similar cytopathology in other target tissues but not in the LO. Mayer-Bennett H&E. 525x and 1700x magnifications, respectively.

C.2.3.1 *Presumptive*

There are no gross observations (Level I) or histopathological (Level II) diagnostic techniques which can provide presumptive detection of YHD in sub-clinical shrimp.

C.2.3.2 *Confirmatory*

C.2.3.2.1 Reverse Transcriptase-Polymerase Chain Reaction Assay (Level III)

For certification of YHV infection status of broodstock and fry, reverse transcriptase-polymerase chain reaction (RT-PCR) technology is recommended.

There are several commercially available RT-PCR kits now available to screen haemolymph from broodstock shrimp and PL tissues for evidence of YHV RNA.

C.2.4 **Diagnostic Methods**

More detailed information on methods for diagnosis can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or at selected references.

C.2.4.1 *Presumptive*

C.2.4.1.1 Gross Observations (Level 1)

YHD can be suspected when an abnormal increase in feeding rates is followed by a sharp cessation in feeding. Moribund shrimp may appear near the surface or edges of grow out ponds and show slow swimming behaviour in response to stimuli. These may also show pale overall body colouration, a yellowish cephalothorax, pale gills and hepatopancreas. YHD should be suspected under such circumstances, especially for *P. monodon*, and samples collected for confirmatory diagnosis.

C.2.4.1.2 Gill Squash (Level II)

Fix whole shrimp, or gill filaments, in Davidson's fixative overnight[2]. Wash gill filament in tap water to remove the fixative and stain with Mayer-Bennett's H&E. Clear in xylene and, using a fine pair of needles (a stereo microscope is helpful), break off several secondary filaments and replace the main filament in xylene for permanent reference storage in a sealed vial. Mount secondary filaments, coverslip and use light pressure to flatten the filaments as much as possible, making them easy to see through. This same procedure can be used on thin layers of subcuticular tissue. Moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions approximately 2 mm in diameter or smaller are presumptive for YHD, along with similar observations from haemolymph smears. As with tissue sections and wet-fixed gill filaments, these slides can be kept as a permanent record.

C.2.4.1.3 Haemolymph Smears (Level II)

Smears that show moderate to high numbers of blood cells with pycnotic and karyorrhexic nuclei, with no evidence of bacteria, can be indicative of early YHD. It is important that no bacteria are present, since these can produce similar haemocyte nucleus changes. Such changes are difficult to see in moribund shrimp because of the loss of blood cells so grossly normal shrimp should be sampled for these signs from the same pond where the moribund shrimp were obtained. The haemolymph is collected in a syringe containing twice the haemolymph volume of 25% formalin or modified Davidson's fixative (*i.e.*, with the acetic acid component replaced by water or formalin). The blood cell suspension is mixed thoroughly in the syringe, the needle removed and a drop placed onto a microscope slide. Smear and air dry the preparation before staining with H&E and eosin or other standard blood stains. Dehydrate, mount and coverslip. The results should be consistent with the gill whole mounts (above) or histopathology of tissue sections, in order to make a presumptive YHD diagnosis.

C.2.4.1.4 Histopathology (Level II)

Fix moribund shrimp from a suspected YHD outbreak in Davidson's fixative and process for standard H&E stain. Most tissues where haemolymp is present may be infected, however, principal sites include the lymphoid organ (Oka organ) (Fig.C.2.3.1.4a,b), hepatopancreatic interstitial cells (not tubule epithelial cells), heart, midgut muscle and connective tissue (but not epithelial cells), stomach sub-cuticulum and gill tissues (Fig.C.2.3.1.4c). Light microscopy should reveal moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions, approximately 2 mm in diameter (smaller in ectodermal and mesodermal tissues). Moribund shrimp show systemic necrosis of gill and stomach sub-cuticular cells, with

[2] If more rapid results are required, fixation can be shortened to 2 hours by substituting the acetic acid component of Davidson's fixative with 50% concentrated HCl (this should be stored **no** more than a few days before use). After fixation, wash thoroughly and check that the pH has returned to near neutral before staining. Do not fix for longer periods or above 25°C as this may result in excessive tissue damage that will make interpretation difficult or impossible.

intense basophilic cytoplasmic inclusions (H&E staining) due to phagocytosed nuclei and viral inclusions. In the lymphoid organ, high numbers of karyorrhexic and pyknotic basophilic inclusions are found in matrix cells of the normal tubules. On the other hand, similar inclusions– are found only in lymphoid organ spheroids with Rhabdovirus of Penaeid Shrimp (RPS) described from Hawaii and Lymphoidal Parvo-like Virus (LPV, LOV) described from Australia; Lymphoid Organ Vacuolisation Virus (LOVV) in *P. vannamei* in Hawaii and the Americas; and Taura Syndrome Virus (TSV) in *P. vannamei, P. stylirostris* and *P. setiferus* from central and south America. Gill Associated Virus (GAV) in Australian *P. monodon*; a Yellow-Head-Disease-Like Virus (YHDLV) in *P. japonicus* from Taiwan Province of China produce similar histopathology to YHV.

C.2.4.2 *Confirmatory*

In cases where results from presumptive screening indicate possible YHD infection, but confirmation of the infectious agent is required (*e.g.*, first time finding or presence of other pathogenic factors), bioassay (see C.2.4.2.1), electron microscopy (see C.2.4.2.2) and molecular techniques (see C.2.4.2.3-5) are required.

C.2.4.2.1 Bioassay (Levels I-II)

The simplest bioassay method is to allow na?ve shrimp (± 10 g wet weight) to feed on carcasses of suspect shrimp. Alternatively, prepare homogenates of gill tissues from suspect shrimp. Centrifuge solids into a loose pellet, decant and filter (0.45 - 0.22 mm) the supernatant. Expose na?ve juvenile *Penaeus monodon* (± 10 g wet weight) to the supernatant Infected shrimp should evoke clinical signs in the na?ve shrimp within 24-72 hours and 100% mortality will generally occur within 3-5 days. Infections should be confirmed by histology of gills and haemolymph.

C.2.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

For TEM, the most suitable tissues of moribund shrimp suspected to be infected by YHD are the lymphoid organ and gills. Fix tissues in 2.5% glutaraldehyde, 2% paraformaldehyde in cacodylate buffer and post-fix in 1% osmium tetroxide, prior to dehydration and embedding in Spurr's resin. 50nm sections are mounted on Cu-200 grids and should be stained with uranyl acetate/70% methanol and Reynold's lead citrate. Diagnosis of YHV is confirmed by the presence of non-occluded, enveloped, rod-shaped particles, 150-200 x 40-50 *nm* in size in the perinuclear or cytoplasmic area of the target tissues or within cytoplasmic vesicles. Non-enveloped, filamentous forms measuring <800 *nm* may also be found in the cytoplasm. The cytoplasm of infected cells becomes fragmented and breaks down within 32 hr of infection.

C.2.4.2.3 Western Blot Assay (Level III)

Remove 0.1 ml of haemolymph from live YHD-suspected shrimp and dilute with 0.1 ml of citrate buffer for immediate use or store at -80oC until examination. A purified viral preparation is required as a positive control, and confirmation is made on the presence of 4 major protein bands characteristic of YHV at 135 and 175 kDa. The sensitivity of the Western blot assay is 0.4 ng of YHV protein.

C.2.4.2.4 Reverse Transcriptase-Polymerase Chain Reaction (Level III)

RT-PCR can be conducted on the haemolymph of suspect shrimp or on post-larvae (see C.2.3.2.1). There are several commercially available RT-PCR kits now available to screen haemolymph from broodstock shrimp and PL tissues for evidence of YHV RNA.

C.2.4.2.5 *In situ* Nucleic Acid Hybridization (Level III)

Commercial *in situ* hybridization kits for YHD are now available.

C.2.5 **Modes of Transmission**

Infections are generally believed to be horizontally transmitted. Survivors of YHD infection, however, maintain chronic sub-clinical infections and vertical transmission is suspected with such individuals. There are a number of known or suspected carrier crustaceans including the brackish water shrimp, *Palaemon styliferus* and *Acetes* sp., which can potentially transmit YHD to farmed shrimp.

C.2.6 **Control Measures**

There are no known treatments for shrimp infected with YHV. However, a number of preventative measures are recommended to reduce spread. These include the following:

- broodstock specimens be screened for YHV

- infected individuals and their offspring be destroyed in a sanitary manner
- associated equipment and rearing water are disinfected
- exclude potential carriers of YHD by screening PL pre-stocking in ponds
- prevention of exposure to potential carriers, post-stocking, can be achieved by filtration or prior treatment in storage ponds of water used for water exchanges.
- avoidance of rapid changes in pH or prolonged periods of low (<2ppm) dissolved oxygen. These can trigger sub-lethal outbreaks of YHD. Alkalinity should not vary more than 0.5 pH units daily and water pH levels > 9 should be avoided. Changes in salinity apparently do not trigger outbreaks.
- avoid fresh aquatic feeds in grow-out ponds, maturation units and hatchery facilities, unless the feed is subjected to prior sterilization (gamma radiation) or pasteurization (*i.e.*, holding at 7˚C for 10 min).

If an outbreak occurs, it is recommended that the affected pond be treated with 30 ppm chlorine to kill the shrimp and potential carriers. The dead shrimp and other animals should be removed and buried or burned. If they cannot be removed, the pond should be thoroughly dried before restocking.

If the outbreak pond can be emergency harvested, the discharge water should be pumped into an adjacent pond for disinfection with chlorine and holding for a minimum of 4 days before discharge. All other waste materials should be buried or burned. Harvesting personnel should change clothing and shower at the site with water that will be discharged into the treatment pond. Clothing used during harvesting should be placed in a specific container to be sent for chlorine treatment and laundering. Equipment, vehicles and rubber boots and the outside of shrimp containers should be disinfected with chlorine and the discharge water run into the treatment pond. Neighbours should be notified of any YHD outbreak and control efforts, and advised not carry out any water exchange for at least 4 days following discharge from the pond used for disinfection. Processing plants receiving emergency harvested shrimp should be notified that the specific lot of shrimp is YHV infected and appropriate measures should be taken at the plant to avoid transfer of the disease via transport containers and processing wastes. Prohibition of introduction of living shrimp from YHV and GAV enzootic areas into historically uninfected areas is recommended.

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C.3 INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS (IHHN)

C.3.1 **Background Information**

C.3.1.1 *Causative Agent*

Infectious Hypodermal and Hematopoietic Necrosis (IHHN) is caused by a non-enveloped icosahedral virus, Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), averaging 22 *nm* in diameter, with a density of 1.40 g/ml in CsCl, containing linear ssDNA with an estimated size of 4.1 kb, and a capsid that has four polypeptides with molecular weights of 74, 47, 39, and 37.5 kD. Because of these characteristics, IHHNV has been classified as a member of the family *Parvoviridae*. More detailed information about the disease can be found at OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a) and Lightner (1996).

C.3.1.2 *Host Range*

IHHNV infects a wide range of penaeid shrimps, but does not appear to infect other decapod crustaceans. Natural infections have been reported in *Penaeus vannamei, P. stylirostris, P. occidentalis, P. monodon, P. semisulcatus, P. californiensis* and *P. japonicus*. Experimental infections have also been reported for *P. setiferus*, *P. aztecus* and *P. duorarum*. *Penaeus indicus* and *P. merguiensis* appear to be refractory to IHHNV infection.

C.3.1.3 *Geographic Distribution*

IHHN occurs in wild and cultured penaeid shrimps in Central America, Ecuador, India, Indonesia, Malaysia, Philippines, Peru, Taiwan Province of China, and Thailand. Although IHHNV has been reported from cultured penaeid shrimp from most regions of the western hemisphere and in wild penaeids throughout their geographic range along the Pacific coast of the Americas (Peru to northern Mexico), it has not been found in penaeids on the Atlantic side of the Americas. IHHNV has been reported in cultured penaeid shrimp from Guam, French Polynesia, Hawaii, Israel and New Caledonia. An IHHN-like virus has also been reported from Australia.

C.3.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease reporting System (1999-2000)*

The disease was suspected in India during the 2nd quarter reporting period for 1999 and 1st quarter reporting period for 2000 (OIE 1999, OIE 2000b).

C.3.2 **Clinical Aspects**

Penaeus stylirostris. Infection by IHHNV causes acute epizootics and mass mortality (> 90%) in *P. stylirostris*. Although vertically infected larvae and early postlarvae do not become diseased, juveniles >35 days old appear susceptible showing gross signs followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease appears size and/or age dependent, with young juveniles always being the most severely affected (Fig.C.3.2a). Infected adults seldom show signs of the disease or mortalities.

Penaeus vannamei. The chronic disease, “runt deformity syndrome” (RDS) (Fig.C.3.2b,c), is caused by IHHNV infection of *P. vannamei*. Juveniles with RDS show wide ranges of sizes, with many smaller than average (“runted”) shrimp. Size variations typically exceed 30% from the mean size and may reach 90%. Uninfected populations of juvenile *P. vannamei* usually show size variations of < 30% of the mean. Similar RDS signs have been observed in cultured *P. stylirostris.*

C.3.3 **Screening Methods**

More detailed information on methods for screening IHHN can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or at selected references.

C.3.3.1 *Presumptive*

There are no gross signs (Level I) or histological features (Level II) that can be used to indicate presumptive infection by IHHNV in sub-clinical carriers.

C.3.3.2 *Confirmatory*

Molecular methods are required to detect IHHNV in sub-clinical carriers.

C.3.3.2.1 Dot Blot Hybridization (Level III)

Haemolymph samples or a small appendage (pleiopod) can be used for dot blot testing. Commercial dot blot hybridization kits for IHHN are now available.

C.3 Infectious Hypodermal And Haematopoietic Necrosis (IHHN)

(DV Lightner)

Fig.C.3.2a. A small juvenile *Penaeus stylirostris* showing gross signs of acute IHHN disease. Visible through the cuticle, especially on the abdomen, are multifocal white to buff colored lesions in the cuticular epithelium or subcutis (arrows). While such lesions are common in *P. stylirostris* with acute terminal IHHN disease, they are not pathognomonic for IHHN disease.

(DV Lightner)

Fig.C.3.2b. Dorsal view of juvenile *P. vannamei* (preserved in Davidson's AFA) showing gross signs of IHHNV-caused RDS. Cuticular abnormalities of the sixth abdominal segment and tail fan are illustrated.

Fig.C.3.4.1.2a. A high magnification of gills showing eosinophilic intranuclear inclusions (Cowdry type A inclusions or CAIs) that are pathognomonic for IHHNV infections. Mayer-Bennett H&E. 1800x magnification.

(DV Lightner)

Fig.C.3.2c. Lateral view of juvenile *P. vannamei* (preserved in Davidson's AFA) showing gross signs of IHHNV-caused RDS. Cuticular abnormalities of the sixth abdominal segment and tail fan are illustrated.

(DV Lightner)

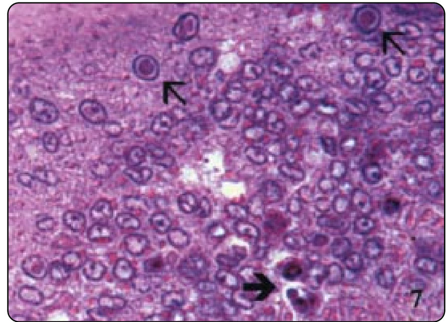
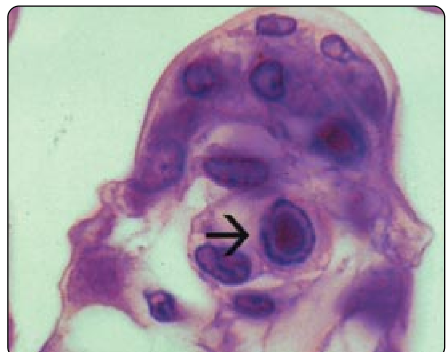


Fig.C.3.4.1.2b. A low magnification photomicrograph (LM) of an H&E stained section of a juvenile *P. stylirostris* with severe acute IHHN disease. This section is through the cuticular epithelium and subcuticular connective tissues just dorsal and posterior to the heart. Numerous necrotic cells with pyknotic nuclei or with pathognomonic eosinophilic intranuclear inclusion bodies (Cowdry type A) are present (arrows). Mayer-Bennett H&E. 830x magnification.

(DV Lightner)



C.3.3.2.2 Polymerase Chain Reaction (PCR) (Level III)

The same tissue samples described in C.3.3.2.1 can be used for non-lethal screening of non-clinical broodstock and juveniles of susceptible species, using PCR.

C.3.4 **Diagnostic Methods**

More detailed information on methods for diagnosis of IHHN can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000b), at http://www.oie.int, or at selected references.

C.3.4.1 *Presumptive*

C.3.4.1.1 Gross Observations (Level I)

Gross signs are not IHHN specific. Acute infections of juvenile *P. stylirostris* may result in a marked reduction in food consumption, followed by changes in behaviour and appearance. The shrimp may rise slowly to the water surface, become motionless and then roll-over, and slowly sink (ventral side up) to the bottom. This behavior may continue for several hours until the shrimp become too weak to continue, or are cannibalised by healthier siblings. By this stage of infection white or buff-coloured spots (which differ from the white spots that occur in WSD - C.4) in the cuticular epidermis, especially at the junction of the abdominal tergal plates, resulting in a mottled appearance. This mottling may later fade in *P. stylirostris*. Moribund *P. stylirostris* may further develop a distinctly bluish colour and opaque abdominal musculature. Although *P. monodon* is frequently found to be infected with IHHNV, it does not generally appear to cause any major clinical disease in the species. Juvenile shrimp (*P. vannamei* and *P. stylirostris*) with RDS display bent or deformed rostrums, wrinkled antennal flagella, cuticular roughness, and other cuticular deformities. They also show a high percentage (30-90%) of stunted growth ("runt shrimp") compared with less than 30% below average size in uninfected populations.

C.3.4.1.2 Histopathology (Level II)

Infected cells occur in the gills (Fig.C.3.4.1.2a), epidermal (Fig.C.3.4.1.2b) and hypodermal epithelia of fore and hindgut, nerve cord and nerve ganglia, as well as mesodermal haematopoietic organs, antennal gland, gonads, lymphoid organ, and connective tissue. Eosinophilic (with H&E stain) intranuclear, Cowdry type A inclusion bodies (CAIs) provide a presumptive diagnosis of IHHNV infection. Infected nuclei are enlarged with a central eosinophilic inclusion sometimes separated from the marginated chromatin by an unstained ringwhen tissues are preserved with acetic acid containing fixatives. Since IHHNV intranuclear inclusion bodies can be confused with developing intranuclear inclusion bodies due to White Spot Disease, electron microscopy (C.3.4.2.2) or *in situ* hybridization assays of suspect sections with IHHNV-specific DNA probes (C.3.4.2.3-5) may be required for definitive diagnosis. Basophilic strands may be visible within the CAIs and cytoplasmic inclusion bodies may also be present.

C.3.4.2 *Confirmatory*

C.3.4.2.1 Bioassay (Levels I/II)

Prevalence and severity of IHHNV infections may be "enhanced" in a quarantined population by holding the suspect shrimp in crowded or other stressful conditions (low dissolved oxygen, elevated water temperature, or elevated ammonia or nitrite). These conditions may encourage expression of low grade IHHNV infections and transmission from sub-clinical carriers to uninfected shrimp. This increase in prevalence and severity can enhance detection using screening methods.

Indicator shrimp (0.1-4.0 gm juvenile *P. stylirostris*) can also be used to assess the presence of IHHNV by cohabitation, feeding of minced carcasses or injection with cell-free homogenates from suspect shrimp.

C.3.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

Negative stain preparations of purified virus show non-enveloped, icosahedral virions, 20-22 nm in diameter. Transmission electron microscopic preparations show intranuclear inclusions containing virions 17-26 *nm* in diameter. Viral particles are also present in the cytoplasm where they assemble and replicate. Chromatin strands (that may be visible as basophilic inclusions under light microscopy) are a prominent feature of IHHNV intranuclear inclusion bodies. Paracrystalline arrays of virions correspond to cytoplasmic inclusion bodies that may be detected under light microscopy.

C.3.4.2.3 Dot Blot Hybridization (Level III)

As described in C.3.3.2.1.

C.3.4.2.2 Polymerase Chain Reaction (Level III)

As described in C.3.3.2.2.

C.3.4.2.5 *In situ* Hybridization (Level III)

IHHNV-specific DNA probes are now available for *in situ* hybridization confirmation of histological and/or electron microscopic observation.

C.3.5 **Modes of Transmission**

Some members of populations of *P. stylirostris* and *P. vannamei*, which survive IHHNV infections and/or epizootics, may carry sub-clinical infections for life which may be passed horizontally to other stocks, or vertically, if used as broodstock.

C.3.6 **Control Measures**

Eradication methods for IHHNV can be applied to certain aquaculture situations. These methods are dependent upon eradication of infected stocks, disinfection of the culture facility, avoidance of re-introduction of the virus (from other nearby culture facilities, wild shrimp, *etc.*), and re-stocking with IHHNV-free post-larvae that have been produced from IHHNV-free broodstock.

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C.4 WHITE SPOT DISEASE (WSD)[3]

C.4.1 **Background Information**

C.4.1.1 *Causative Agent*

The causative agent of white spot disease (WSD) is the white spot syndrome virus (WSSV) or white spot virus (WSV), a double stranded DNA (dsDNA) virus. In initial reports, WSV was described as a non-occluded baculovirus but subsequent analysis of WSV-DNA sequences does not support this contention. The viruses in this complex have recently been shown to comprise a new group with the proposed name of Nimaviridae (Van Hulten *et al.* 2001). In the literature, however, several names have been used to describe the virus, including baculoviral hypodermal and haematopoietic necrosis (HHNBV), Shrimp Explosive Epidemic Disease (SEED), China virus disease, rod-shaped nuclear virus of *Penaeus japonicus* (RV-PJ); systemic ectodermal and mesodermal baculovirus (SEMBV), white spot baculovirus (WSBV) and white spot syndrome virus (WSSV). More detailed information about the disease can be found in the OIE Manual for Aquatic Animal Diseases (OIE 2000a) and Lightner (1996).

C.4.1.2 *Host Range*

White spot disease has a wide spectrum of hosts. Outbreaks were first reported from farmed *Penaeus japonicus* in Japan and natural infections have subsequently been observed in *P. chinensis*, *P. indicus*, *P. merguiensis*, *P. monodon, P. setiferus, P. stylirostris, and P. vannamei.* In experimental studies, WSD is also lethal to *P. aztecus, P. duodarum* and *P. setiferus.*

C.4.1.3 *Geographic Distribution*

WSD was first reported in Taiwan Province of China and China mainland between 1991-1992, and in Japan in 1993 from shrimp imported from China PR. Later outbreaks have been reported from elsewhere in Asia including China PR, India, Indonesia, Korea RO, Malaysia, Taiwan Province of China, Thailand, and Vietnam. In addition to the Asian countries listed above, farmed shrimp exhibiting the gross signs and histology of WSD have been reported in the USA and Latin America.

As of 1999, WSD has been reported in at least nine countries in the Americas: Columbia, Ecuador, Guatemala, Honduras, Mexico, Nicaragua, Panama, Peru and USA (Subasinghe *et al.* 2001).

C.4.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

WSD was reported by Bangladesh, China PR, India, Indonesia, Japan, Korea RO, Malaysia, Philippines, Taiwan Province of China, Sri Lanka, and Thailand; and suspected in Pakistan during the reporting period for the year 1999. In year 2000, Bangladesh, India, Japan, Korea RO, Malaysia, Philippines, Sri Lanka, Thailand and Vietnam reported positive occurrence of the disease (NACA/FAO 2000a,b,c; OIE 1999, OIE 2000a,b).

C.4.2 **Clinical Aspects**

WSD outbeaks are often characterised by high and rapid mortality of infected populations, usually shortly after the first appearance of the clinical signs. Acutely affected shrimp demonstrate anorexia and lethargy, have a loose cuticle with numerous white spots (about 0.5 to 2.0 mm in diameter) on the inside surface of the carapace (Fig.C.4.2a,b). These spots are within the cuticle structure and cannot be removed by scraping. Moribund shrimp may also show a pink to red discolouration. Susceptible shrimp species displaying these clinical signs are likely to undergo high levels of mortality. Pathology is associated with systemic destruction of the ectodermal and mesodermal tissues of the gills and sub-cuticular tissues.

C.4.3 **Screening Methods**

More detailed information on methods for screening for WSD can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or in selected references.

C.4.3.1 *Presumptive*

There are no gross observations (Level I) or histopathological (Level II) diagnostic techniques which can provide presumptive detection of WSD in sub-clinical shrimp.

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[3] White spot disease (WSD) is now classified as an OIE Notifiable Disease (OIE 2000a).

(DV Lightner)

Fig.C.4.2a. A juvenile *P. monodon* with distinctive white spots of WSD.

(DV Lightner/P. Saibaba)

Fig.C.4.2b. Carapace from a juvenile *P. monodon* with WSD. Calcareous deposits on the underside of the shell account for the white spots.

C.4.3.2 *Confirmatory*

C.4.3.2.1 Nested PCR of Tissues and Haemolymph (Level III)

The protocol described by Lo *et al* (1996, 1998) is the recommended procedure for nested PCR of tissues and haemolymph. There are also commercially available kits for detection of WSD in sub-clinical carriers using PCR-based techniques.

C.4.3.2.2 Polymerase Chain Reaction (PCR) of Postlarvae (Level III)

From a nursery or hatchery tank containing 100 000 postlarvae (PL) or more, sample approximately 1000 PL from each of 5 different points.

(DV Lightner)

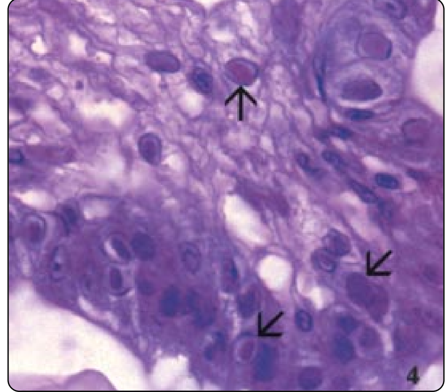

Fig.C.4.3.3.1.2a. Histological section from the stomach of a juvenile *P.chinensis* infected with WSD. Prominent intranuclear inclusion bodies are abundant in the cuticular epithelium and subcuticular connective tissue of the organ (arrows).

(DV Lightner)

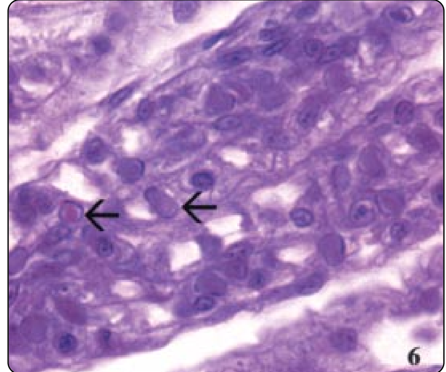

Fig.C.4.3.3.1.2b. Section of the gills from a juvenile *P. chinensis* with WSBV. Infected cells show developing and fully developed intranuclear inclusion bodies of WSBV (arrows). Mayer-Bennett H&E. 900x magnification.

Pool the samples in a basin, gently swirl the water and select an assay sample from living PL collected at the center of the basin. A sample of 150 PL is required to give a 95% confidence of detecting an infection at 2% prevalence in the population (see Table C.1.3.3 of C.1 General Techniques).

For PL 11 and older, exclude shrimp eyes from any tissue samples, since these inhibit the PCR process. Follow the procedures from the recommended source for nested PCR given under C.4.3.2.1.

C.4.3.2.3 Dot Blot Hybridization (Level III)

Details on dot blot hybridisation techniques and detection kit availability are provided in the OIE Diagnostic Manual (OIE 2000a).

C.4.3.2.4 *In situ* Hybridization (Level III)

Details on *in situ* hybridization techniques and detection kit availability are provided in the OIE Diagnostic Manual (OIE 2000a).

C.4.4 **Diagnostic Methods**

More detailed information on methods for diagnosis of WSD can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or in selected references.

C.4.4.1 *Presumptive*

C.4.4.1.1 Gross Observations (Level I)

WSD outbreaks are generally preceded by cessation of feeding followed, within a few days, by the appearance of moribund shrimp swimming near the surface at the edge of rearing ponds. These shrimp exhibit white inclusions embedded in the cuticle and often show reddish discolouration of the body. The cuticular inclusions range from minute spots to discs several mm in diameter that may coalesce into larger plaques. They are most easily observed by removing the cuticle from the cephalothorax, scraping away any attached tissue and holding the cuticle up to the light. The appearance of white spots in the cuticle can be caused by other conditions. In particular, Wang *et al.*, 2000, report a condition called bacterial white spot syndrome (BWSS) which can easily be mistaken for WSD (see C.4a). Therefore, histopathological examination is required for confirmatory diagnosis.

C.4.4.1.2 Rapid Squash Mount Preparations (Level II)

Two types of rapid squash mount preparations that can be used for presumptive diagnosis of WSD: i) fresh, unstained wet mounts fixed in 10% formalin solution and viewed by dark field microscopy with a wet-type condenser, and ii) fixed tissues stained with H&E.

For method ii) fix whole shrimp or gill filaments in Davidson's fixative overnight. If more rapid results are required, fixation can be shortened to 2 hrs by changing the acetic acid in the Davidson's fixative to 50% concentrated HCl (this should not be stored longer than a few days before use). After fixation, wash the tissues thoroughly and ensure pH is near neutral before staining. Do not fix for longer periods, or above 25°C, as this can cause tissue damage that will make interpretation difficult or impossible. Stain with Meyer's H&E and dehydrate to xylene (or equivalent clearing solution). Place a gill filament on a microscope slide tease off several secondary filaments. Replace the main filament in a sealed vial filled with xylene as a permanent back-up reference. Being careful not to let the secondary gill filaments dry, tease apart and remove any large fragments or particles from the slide. Add a drop of mounting fluid and a cover glass, using light pressure to flatten the tissue as much as possible. The same procedure can be used for thin layers of subcuticular tissue.

Examine under a compound microscope at 40x magnification for moderate to large numbers of hypertrophied nuclei with basophilic, centrally-positioned, inclusions surrounded by marginated chromatin. The whole mount slides can also be kept as permanent records.

C.4.4.1.3 Histopathology (Level II)

Moribund shrimp from a suspected WSD outbreak should be fixed in Davidson's fixative and stained with haematoxylin and eosin (H&E). The histopathology of WSD is distinctive, and can provide a conclusive diagnosis. However, first time detection or detection in species not previously reported to be susceptible, require molecular assay or electron microscopy demonstration of a viral aetiology.

Moribund shrimp with WSV show systemic destruction of ectodermal and mesodermal tissues. Nuclei of infected cells are hypertrophied and when stained with haematoxylin and eosin show lightly to deeply basophilic central inclusions surrounded by marginated chromatin. These intranuclear inclusions can also be seen in squash mounts of gills or sub-cuticular tissue (see C.4.4.1.2), or in tissue sections. The best tissues for examination are the subcuticular tissue of the stomach (Fig.C.4.3.3.1.2a), cephalothorax or gill tissues (Fig.C.4.3.3.1.2b).

C.4.4.2 *Confirmatory*

A definitive diagnosis can be accomplished by polymerase chain reaction (PCR) technology

(single-step or nested), *in situ* hybridization, Western blot analysis (detailed protocols can be found in OIE (2000a) or electron microscopy (TEM).

C.4.4.2.5 Transmission Electron Microscopy (TEM) (Level III)

The most suitable tissues for TEM examination are subcuticular tissues, gills and pereiopods that have been pre-screened by histology (C.4.4.1.3) or rapid-stain tissue squashes (C.4.4.1.2) which show signs of hypertrophied nuclei with Cowdry A-type inclusions or marginated chromatin surrounding a basophilic inclusion body. Fix tissues for at least 24h in a 10:1 fixative to tissue volume ration of 6% gluteraldehyde at 4°C and buffered with sodium cacodylate or phosphate solution to pH7. For longer term storage, reduce gluteraldehyde to 0.5-1.0% concentration. Post-fix in 1% osmium tetroxide, and stain with uranyl acetate and lead citrate (or equivalent TEM stain). WSD virions are rod-shaped to elliptical with a trilaminar envelope and measure 80-120 x 250-380 *nm*.

C.4.4.2.6 Negative Stain Electron Microscopy (Level III)

Negative stain preparations from shrimp haemolymph may show virions with unique, tail-like appendages within the hypertrophied nuclei of infected cells, but no evidence of occlusion bodies.

C.4.5 **Modes of Transmission**

Wild broodstock and fry used to stock rearing ponds are known to carry WSV, as are numerous other crustaceans and even aquatic insect larvae. Molecular techniques have been used to confirm infection of non-penaeid carriers of WSV and transmission studies show that these can transmit WSV to shrimp.

C.4.6 **Control Measures**

There are no known treatments for shrimp infected with WSV, however, a number of preventative measures are recommended to reduce spread.

At facilities used for the production of PL, it is recommended that wild broodstock be screened for WSD by nested PCR. Any infected individuals, and their offspring, should be destroyed in a sanitary manner and all contaminated equipment and rearing water be disinfected. It is also recommended that broodstock *P. monodon* be tested for WSD after spawning to increase the probability of viral detection.

At grow-out, PL should be screened for freedom from WSV by nested PCR using sufficiently large numbers of PL to ensure detection of significant infections. A biased sampling regime, which selects weaker animals for testing, can further increase the probability of detecting infected batches.

During cultivation, it is suspected that rapid changes in water temperature, hardness and salinity, or reduced oxygen levels (<2 ppm) for extended periods, can trigger outbreaks of WSD in shrimp with sub-clinical infections. It is not yet known whether large diurnal pH changes can trigger outbreaks but stable pond-water pH is known to reduce general stress levels in shrimp. Fresh or fresh-frozen feeds of aquatic animal origin should *not* be used in the grow-out ponds, maturation units and hatchery facilities unless subjected to prior sterilization (gamma radiation) or pasteurization (*i.e.*, holding at 70°C for 10 min).

Any affected ponds should be treated immediately with 30 ppm chlorine to kill the infected shrimp and any potential carriers. The dead shrimp and other animals should be removed and buried or burned. The water should then be held for a minimum of 4 days before discharge. Neighbouring pond owners should be immediately informed and should not carry out water exchange for a minimum of 4 days after water is discharged from an outbreak pond if it is likely to come into contact with their own supply water.

If the outbreak pond is emergency harvested, the discharge water should be pumped into an adjacent pond or reservoir for disinfection with chlorine and holding for a minimum of 4 days before discharge. All water from the harvested pond should be discharged into the treatment pond and any waste materials should be buried or burned. Harvesting personnel should change clothing and shower at the site with water that will be discharged into the treatment pond. Clothing used during harvesting should be placed in a specific container to be sent for disinfection and laundering. Equipment, vehicles, footwear and the outside of shrimp containers should be disinfected and the waste water discarded into the treatment pond. The processing plant should be notified that the specific lot of shrimp is WSD infected and appropriate measures should be taken at the plant

to avoid transfer of the disease via transport containers and processing wastes. Prevention of introduction of live shrimp from WSV enzootic areas into historically uninfected areas or areas defined as free from the disease is recommended.

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C.4a BACTERIAL WHITE SPOT SYNDROME (BWSS)

Bacterial White Spot Syndrome (BWSS) is a recently described condition which affects *Penaeus monodon*. It is, as yet, poorly understood condition and is included in the Asia Diagnostic Guide due to the possibility of diagnostic confusion with viral White Spot Disease (WSD).

C.4a.1 **Background Information**

Since 1993, white spot disease virus (WSDV) has caused massive losses to the shrimp industry in Asia and Latin America. Recently, another disease syndrome showing similar gross clinical signs of white spots, has been detected and reported as "bacterial white spot syndrome'' (BWSS) (Wang *et al.*, 1999*,* 2000). The similar gross clinical signs have also caused confusion during PCR-based screening for WSD since, shrimp with apparent WSDV clinical signs, give negative results. The clinical effects of BWSS, appear far less significant than those of WSD infection, although it has been suggested that severe infections may reduce moulting and growth.

C.4a.1.1 *Causative Agent(s)*

The bacterium *Bacillus subtilis* has been suggested as the possible causative agent due to its association with the white spots (Wang *et al.*, 2000) but no causal relationship has been demonstrated, nor have infectivity studies been conducted. *Vibrio cholerae* is also often isolated in significant numbers and similar white spots have been described in farmed shrimp in Thailand as a result of exposure to high pH and alkalinity in ponds in the absence of the White Spot virus or bacterial colonisation of the spots, indicating that the bacterial involvement may be secondary. The lack of certainty as to the causative agent and the possibility of secondary involvement of bacteria needs to be addressed through further research. Until the bacterial etiology is clearly demonstrated, bacteria cannot be definitively regarded as the causative agent.

C.4a.1.2 *Host Range*

To date, the syndrome has only been reported in cultured *Penaeus monodon*.

C.4a.1.3 *Geographic Distribution*

BWSS was first detected from a shrimp (*Penaeus monodon)* farm in Malaysia in 1998 (Wang *et al*. 1999, 2000). This remains the only confirmed report of the condition.

C.4a.2 **Clinical Aspects**

Dull white spots are seen on the carapace and all over the body but are more noticeable when the cuticle is peeled away from the body. The white spots are rounded and not as dense as those seen in WSD (Fig.C.4a.2). Wet mount microscopy reveals the spots as opaque brownish lichen-like lesions with a crenellated margin (although this is also the case with spots in the early stages of WSD and cannot be used as a distinctive diagnostic feature). The spot center is often eroded and even perforated. During the early stage of infection, shrimp are still active, feeding and able to moult – at which point the white spots may be lost. However, delayed moulting, reduced growth and low mortalities have been reported in severely infected shrimp (Wang *et al.*, 2000).

C.4a.3 **Screening Methods**

There are no reported methodologies available to screen for sub-clinical infections, since BWSS appears to be an opportunistic infection.

C.4a.4 **Diagnostic Methods**

C.4a.4.1 *Presumptive*

C.4a.4.1.1 Gross Observations (Level I)

The presence of white spots on shrimp cuticles without significant mortality.

C.4a.4.1.2 Wet Mounts (Level I)

If cuticular spots are detected in *P. monodon*, which show an opaque brownish lichen-like appearance with a crenallated margin and the center shows signs of erosion and/or perforation, along with extensive bacterial involvement, such infections could be attributable to BWSS. Such infections should be confirmed as being negative for WSD.

C.4a.4.1.2 Polymerase Chain Reaction (PCR) (Level III)

Negative WSDV-PCR results from samples showing gross clinical signs attributed to WSD, may be suggestive of the alternate aetiology of BWSS.

C.4a Bacterial White Spot Syndrome (BWSS)

(M. Shariff)

Fig. C.4a.2. *Penaeus monodon* dense white spots on the carapace induced by WSD.

(M. Shariff/ Wang *et al.* 2000 (DAO 41:9-18))

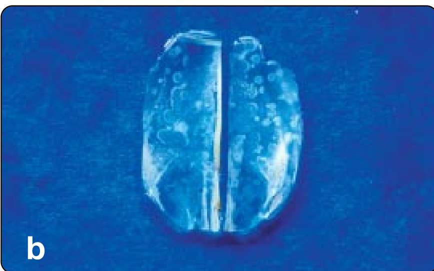
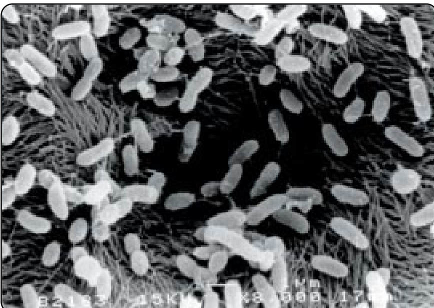


Fig. C.4a.4.2.2a, b. Bacterial white spots (BWS), which are less dense than virus-induced white spots. Note some BWS have a distinct whitish marginal ring and maybe with or without a pinpoint whitish dot in the center

(M. Shariff/ Wang *et al.* 2000 (DAO 41:9-18))



C.4a.4.2 *Confirmatory*

C.4a.4.2.1 Histopathology (Level II)

Histological examinations should be conducted to ensure that the soft-tissues associated with the cuticular lesions do *not* show signs of the WSDV characteristic endodermal and mesodermal intranuclear inclusian bodies. In the case of BWSS, bacteria will be the primary microbial foreign particle and this should be in primary association with the cuticular lesions themselves.

C.4a.4.2.2 Scanning Electron Microscopy (TEM) (Level III)

The presence of spot lesions (Fig. C.4a.4.2.1a,b) together with numerous bacteria (Fig. C.4a.4.2.2c) under scanning electron microscopy will confirm BWSS.

C.4a.5 **Modes of Transmission**

Since bacteria are only localized on the body surface, the mode of transmission is thought to be through the rearing water. However, this has yet to be demonstrated using transmission studies.

C.4a.6 **Control Measures**

Although the exact aetiology is unknown, some measures may help to reduce the risk of BWSS. Build up of high bacterial density in rearing water should be avoided. Changing water frequently is recommended . Indiscriminate use of probiotics containing *Bacillus subtilis* should also be avoided until the relationship between this bacteria and the BWSS syndrome is better understood. It has been claimed that BWSS in shrimp ponds can be treated with quick lime (CaO) at 25 ppm, however, this is still under investigation and the use of quicklime may itself cause problems due to rapid increases in pond-water pH (see C.4.6).

Fig. C.4a.4.2.2c. Presence of large number of bacteria attached to exposed fibrillar laminae of the endocuticle.

C.4a Bacterial White Spot Syndrome (BWSS)

C.4a.7 **Selected References**

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Wang, Y. G., K.L. Lee, M. Najiah, M. Shariff and M.D. Hassan. 2000. A new bacterial white spot syndrome (BWSS) in cultured tiger shrimp *Penaeus monodon* and its comparison with white spot syndrome (WSS) caused by virus. *Dis. Aquat. Org.* 41: 9-18.

C.5 BACULOVIRAL MIDGUT GLAND NECROSIS (BMN)

C.5.1 Background Information

C.5.1.1 *Causative Agent*

The pathogen responsible for Baculoviral Midgut Gland Necrosis (BMN) disease is Baculoviral midgut gland necrosis virus (BMNV), a non-occluded gut-infecting baculovirus, whose non-enveloped nucleocapsid measures 36 by 250 *nm*; enveloped virions measures ~ 72 by ~ 310 *nm*. More detailed information about the disease can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (2000a), and Lighter (1996).

C.5.1.2 *Host Range*

BMN was observed as natural infections in *Penaeus japonicus*, *P. monodon* and *P. plebejus* (Fig.C.5.1.2a); and as experimental infections in *P. chinensis* and *P. semisulcatus*.

C.5.1.3 *Geographic Distribution*

BMN has occurred in the Kyushu and Chugoku area of Japan since 1971. BMN-like virus (non-occluded, type C baculovirus) has also been reported in *P. japonicus* in Korea RO and from *P. monodon* in the Philippines and possibly in Australia and Indonesia.

C.5.1.4 *Asia-Pacific Quarterly Aquatic Animal Diseases Reporting System (1999-2000)*

For the reporting year 1999, no positive report from Japan (1992 was last year of occurrence). The disease was suspected in Korea RO from January to September 1999 and whole year of 2000 (OIE 1999, OIE 2000a).

C.5.2 Clinical Aspects

In Japan, BMN is considered to be one of the major problems in hatcheries where it infects larvae and early postlarval stages causing high mortalities. The apparent white turbidity of the hepatopancreas is caused by necrosis of hepatopancreas tubule epithelium and possibly also the mucosal epithelium. Larvae float inactively but later stages (late PL) tend to show resistance to the disease.

C.5.3 Screening Methods

More detailed information on methods for screening BMN can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or at selected references.

C.5.3.1 *Presumptive*

Techniques suitable for presumptive screening of asymptomatic animals at Levels I or II are not available.

C.5.3.2 *Confirmatory*

C.5.3.2.1 Histopathology (Level II)

Histopathology as described for C.5.4.2.1 is the standard screening method recommended by OIE (2000a).

C.5.4 Diagnostic Methods

More detailed information on methods for diagnosis can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or at selected references.

C.5.4.1 *Presumptive*

C.5.4.1.1 Gross Observations (Level 1)

Morbid or larvae heavily infected with BMNV shows a cloudy midgut gland, easily observable by the naked eye.

C.5.4.1.2 Wet-Mount Technique (Level II)

Hypertrophied nuclei in fresh squashes (viewed under dark-field microscopy) or in stained smears of hepatopancreas (using light microscopy) are demonstrated in BMNV infected samples. When viewed under dark-field illumination equipped with a wet-type condenser, the infected nuclei appear white against the dark background. This is due to the increased reflected and diffracted rays produced by numerous virus particles in the nucleus. Samples fixed in 10% formalin also give same results.

C.5.4.2 *Confirmatory*

C.5.4.2.1 Histopathology (Level II)

Samples are fixed in Davidson's fixative, stained with standard H&E and examined under bright field microscopy. Infected shrimps show greatly hypertropied nuclei (Fig.C.5.4.2.1a) in hepatopancreatic epithelial cells undergoing necrosis. Infected nuclei show diminished nuclear chromatin, marginated chromatin (Fig. C.5.4.2.1b, c) and absence of occlusion bodies characteristic of *Baculovirus penaei* (BP) (see also Fig. C.9.3.2.3a,b – section C.9) and

C.5 Baculoviral Midgut Gland Necrosis (BMN)

(DV Lightner)

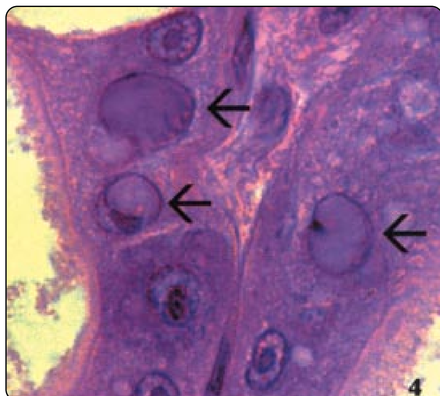


Fig.C.5.1.2a. Section of the hepatopancreas of *P. plebejus* displaying several hepatopancreas cells containing BMN-type intranuclear inclusion bodies. Mayer-Bennett H&E. 1700 x magnification.

(DV Lightner)

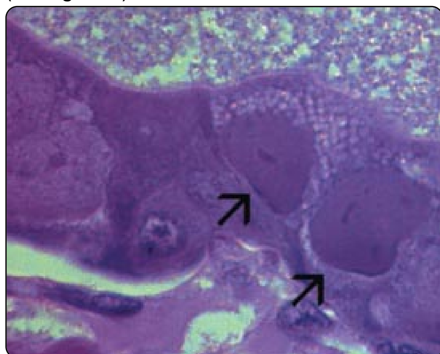
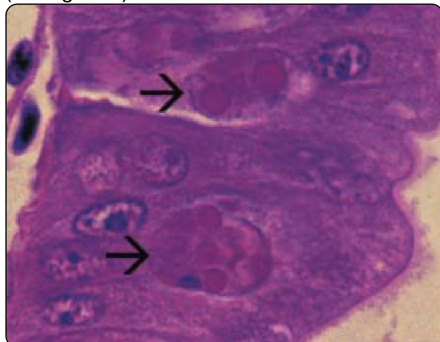
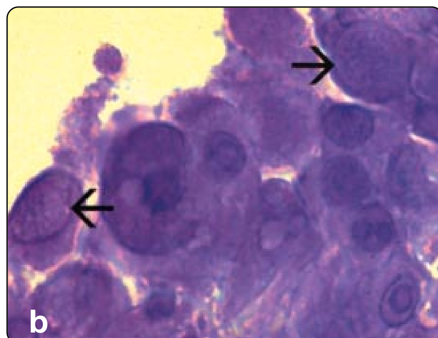


Fig.C.5.4.2.1a. High magnification of hepatopancreas from a PL of *P. monodon* with a severe infection by a BMN-type baculovirus. Most of the hepatopancreas cells display infected nuclei. Mayer-Bennett H&E. 1700x magnification.

(DV Lightner)



(DV Lightner)



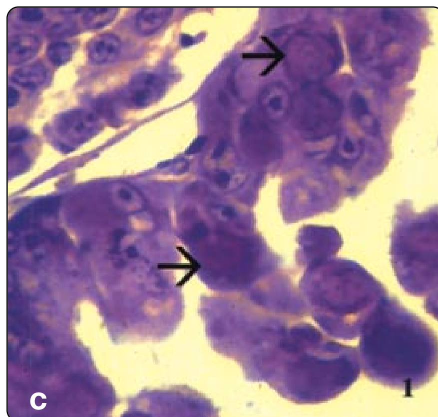


Fig. C.5.4.2.1b, c. Sections of the hepatopancreas of a PL of *P. japonicus* with severe BMN. Hepatopancreas tubules are mostly destroyed and the remaining tubule epithelial cells contain markedly hypertrophied nuclei that contain a single eosinophilic to pale basophilic, irregularly shaped inclusion body that fills the nucleus. BMNV infected nuclei also display diminished nuclear chromatin, marginated chromatin and absence of occlusion bodies that characterize infections by the occluded baculoviruses. Mayer-Bennett H&E. Magnifications: (a) 1300x; (b) 1700x.

Fig.C.5.4.2.1d. MBV occlusion bodies which appear as esosinophilic, generally multiple, spherical inclusion bodies in enormously hypertrophied nuclei (arrows). Mayer-Bennett H&E. 1700x magnification.

C.5 Baculoviral Midgut Gland Necrosis (BMN)

Monodon Baculovirus (MBV) infections (Fig.C.5.4.2.1d).

C.5.4.2.2 Transmission Electron Microscopy (TEM) (Level III)

Tranmission electron microscopy can be used confirm diagnosis of BMN through demonstration of the rod-shaped enveloped virions as described in C.5.1.1.

C.5.4 **Modes of Transmission**

The oral route has been demonstrated to be the main infection pathway for BMNV infection. Viruses released with faeces into the environmental water of intensive culture systems of *P. japonicus* play an important role in disease spread.

C.5.5 **Control Measures**

The concentrations of various disinfectants required to kill BMNV are toxic to shrimp larvae. Complete or partial eradication of viral infection may be accomplished by thorough washing of fertile eggs or nauplii using clean sea water to remove the adhering excreta. Disinfection of the culture facility and the avoidance of re-introduction of the virus are critical factors to control BMN disease.

The suggested procedure for eradication of BMN infection involves collection of fertile eggs from broodstock and passing them through a soft gauze with pore size of 800 mm to remove digested excrement or faeces of the shrimp. The eggs are then washed with running sea water at salinity level of 28-30% for 3-5 min to make sure all the faecal debris has been removed. The eggs are then collected by passing the suspension through a soft gauze with pore size of 100 mm. The eggs are then further washed with running sea water at salinity level of 28-30% for 3-5 min to remove the adhesive viral particles.

C.5.6 **Selected References**

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C.6 GILL-ASSOCIATED VIRUS (GAV)

C.6.1 **Background**

C.6.1.1 *Causative Agent*

Gill-associated virus (GAV) is a single-stranded RNA virus related to viruses of the family *Coronaviridae.* It is closely related to yellow head virus and is regarded as a member of the yellow head complex. GAV can occur in healthy or diseased shrimp and was previously called lymphoid organ virus (LOV) when observed in healthy shrimp.

C.6.1.2 *Host Range*

Natural infection with GAV has only been reported in *Penaeus monodon* but experimental infection has caused mortalities in *P. esculentus, P. merguiensis* and *P. japonicus*. An age or size related resistance to disease was observed in *P. japonicus.*

C.6.1.3 *Geographic Distribution*

GAV has only been recorded from Queensland on the north-east coast of Australia and is endemic to *P. monodon* in this region.

C.6.1.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

Australia reported widespread occurrence of LOV among healthy farmed and wild *P. monodon* in Queensland. Other countries reported "no information available" for GAV for the reporting period for 1999 and 2000 (OIE 1999, OIE 2000).

C.6.2 **Clinical Aspects**

GAV is endemic in healthy *P. monodon* in northern Queensland. It is unclear whether the onset of disease results from environmental stress leading to clinical expression of the pre-existing virus as can occur with YHD and WSD or whether the disease arises from a new infection with a pathogenic strain of GAV. GAV is predominantly found in the gill and lymphoid organ but has also been observed in haemocytes. During acute infections, there is a rapid loss of haemocytes, the lymphoid organs appear disorganised and devoid of normal tubule structure, and the virus is detected in the connective tissues of all major organs.

C.6.3 **Screening Methods**

C.6.3.1 *Confirmatory*

C.6.3.1.1 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Level III)

The PCR primers below are designed to amplify a 618 bp region of GAV:

GAV-5 5'-AAC TTT GCC ATC CTC GTC AC-3'
GAV-6 5'-TGG ATG TTG TGT GTT CTC AAC-3'

The PCR primers below are designed to amplify a 317 bp region internal to the region amplified by GAV5 and GAV6:

GAV-1 5'-ATC CAT ACT ACT CTA AAC TTC C-3'
GAV-2 5'-GAA TTT CTC GAA CAA CAG ACG-3'

Total RNA (100 ng) is denatured in the presence of 35 pmol of each primer (GAV-5 and GAV-6) by heating at 98°C for 8 min in 6 ml DEPC-water containing 0.5 ml deionised formamide and quenched on dry ice. cDNA is synthesised by the addition of 2 ml Superscript II buffer x 5, 1 ml 100 mM DTT, 0.5 ml 10 mM dNTPs, 20 U rRNasinTM (Promega) and 100 U Superscript II Reverse Transcriptase (Life Technologies) and DEPC-water to 10 ml and the reaction is incubated at 42°C for 1 hr followed by heating at 99°C for 5 min before quenching on ice. One tenth of the cDNA reaction (1 ml = 10 ng RNA) is amplified in 50 ml using *Taq* buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 35 pmol each primer GAV-5 and GAV-6 and 200 mM dNTPs overlaid with 50 ml liquid paraffin. PCRs are initiated using a "hot-start" protocol in which the reaction was heated at 85oC for 5 min prior to the addition of 2.5 U *Taq* polymerase (Promega). DNA is amplified by 30 cycles of 95oC/1 min, 58°C/1 min, 72°C/40 sec followed by 72°C/10 min final extension and 20oC hold using either a Corbett Research or Omnigene (Hybaid) thermal cycler. PCR products (10 ml) are resolved in 2% agarose-TAE gels containing 0.5 mg/ml ethidium bromide.

When the result of the primary RT-PCR is negative or inconclusive, 0.5 ml of the primary PCR is amplified by nested PCR as above in a 50 ml reaction volume using primers GAV-1 and GAV-2. In some cases, 5 ml of the RT-PCR is used. Nested PCR conditions are as for the primary PCR except that the extension time is reduced to 30 sec and number of cycles is reduced to 20. Nested PCR aliquots (10 ml) are analysed in 2% agarose-TAE gels.

C.6 Gill-Associated Virus (GAV)

C.6.4 **Diagnostic Methods**

C.6.4.1 *Presumptive*

C.6.4.1.1 Gross Observations (Level I)

Shrimp with an acute GAV infection demonstrate lethargy, lack of appetite and swim on the surface or around the edge of ponds. The body may develop a dark red colour particularly on the appendages, tail fan and mouth parts; gills tend to be yellow to pink in colour. Barnacle and tube worm attachment together with gill fouling have also been observed. The gross signs of acute GAV infection are variable and not always seen and thus, they are not reliable, even for preliminary diagnosis.

C.6.4.1.2 Cytology/Histopathology (Level II)

The cephalothorax of infected prawns is separated from the abdomen and split longtudinally. The sample is then fixed in Davidson's fixative and processed for histology. Sections are stained with H&E. Lymphoid organs from diseased shrimp display loss of the normal tubule structure. Where tubule structure is disrupted, there is no obvious cellular or nuclear hypertrophy, pyknotic nuclei or vacuolization. Foci of abnormal cells are observed within the lymphoid organ and these may be darkly eosinophilic. The gills of diseased shrimp display structural damage including fusion of gill filament tips, general necrosis and loss of cuticle from primary and secondary lamellae. The cytology of the gills appears normal apart from small basophilic foci of necrotic cells.

C.6.4.2 *Confirmatory*

C.6.4.2.1 Transmission Electron Microscopy (TEM) (Level III)

Tissue samples are fixed in 2.5% glutaraldehyde/2% paraformaldehyde in cacodylate buffer and post-fixed in 1% osmium tetroxide. Fixed samples are then dehydrated through a graded series of ethanol concentrations and mounted in Spurr's resin. 50 *nm* sections are mounted on Cu-200 grids, stained with uranyl acetate/70% methanol and Reynold's lead citrate. The cytoplasm of lymphoid organ cells from diseased shrimps contains both rod-shaped enveloped virus particles and viral nucleocapsids. The nucleocapsids are from 166-435 *nm* in length 16-18 *nm* in width.

(P Walker)

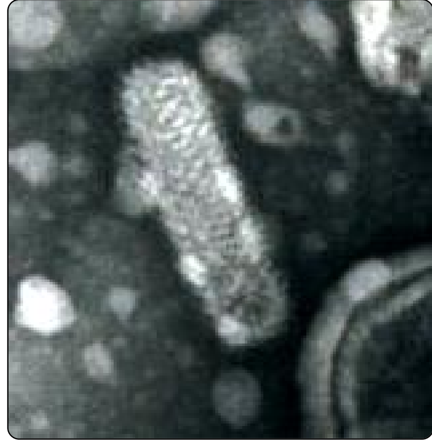

Fig. C.6.4.2.1. Transmission electron microscopy of GAV.

Nucleocapsids have striations with a periodicity of 7 *nm* and are often found associated with the endoplasmic reticulum. Enveloped virions are less common, occurring in about 20% of cells within the disrupted areas of the lymphoid organ. The enveloped virions (Fig. C.6.4.2.1) are 183-200 *nm* long and 34-42 *nm* wide again associated with the endoplasmic reticulum. Both enveloped virions and nucleocapsids are present in gill tissue but the nucleocpsids are more commonly occurring in 40-70% of cells whereas enveloped virions are present in less than 10% of cells.

C.6.4.2.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Level III)

As described for C.6.3.1.1.

C.6.5 **Modes of Transmission**

The most effective form of horizontal transmission is direct cannibalism but transmission can also be water-borne. GAV is also transmitted vertically from healthy broodstock. The virus may be transmitted from either or both parents but it is not clear if infection is within the egg.

C.6.6 **Control Measures**

There are no known control measures for GAV. Prevention of the movement of GAV infected stock into historically uninfected areas is recommended. Drying out of infected ponds appears effective in preventing persistence of the virus.

C.6.7 Selected References

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C.7 SPAWNER-ISOLATED MORTALITY VIRUS DISEASE (SMVD)[4]

C.7.1 **Background Information**

C.7.1.1 *Causative Agent*

Spawner-isolated Mortality Virus Disease (SMVD) is caused by a single-stranded icosahedral DNA virus measuring 20-25 *nm.* These characteristics are most closely associated with those of the Family Parvoviridae. The virus has been named Spawner-isolated Mortality Virus (SMV) and other disease names include Spawner Mortality Syndrome (SMS) and Midcrop Mortality Syndrome (MCMS). More detailed information about the disease can be found in OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a).

C.7.1.2 *Host Range*

SMVD affects *Penaeus monodon*. Experimental infections have also resulted in mortalities of *P. esculentus*, *P. japonicus*, *P. merguiensis* and *Metapenaeus ensis*. Moribund, farmed freshwater crayfish (*Cherax quadricarinatus*) have also been associated with putative SMV infection using DNA-probe analyses.

C.7.1.3 *Geographic Distribution*

SMVD has been reported from Queensland, as well as the Philippines and Sri Lanka.

C.7.1.3.4 *Asia-Pacific Quarterly Aquatic Animal Disease Reporting System (1999-2000)*

Most countries reported "no information available" or "never reported" for the 2 year reporting period (1999 and 2000) except for Sri Lanka which suspected the disease in August 1999 and reported positive occurrence in September 1999 (OIE 1999, OIE 2000b). Philippines reported positive occurrence of SMV in October to December 1998 where samples of *P. monodon* sent to Australia for *insitu* hybridization using SMV probe produced positive results (NACA/FAO 1999).

C.7.2 **Clinical Aspects**

There are no specific clinical signs known for SMV. It is one of several viruses associated with mid-crop mortality syndrome (MCMS) which resulted in significant mortalities of juvenile and sub-adult *P. monodon* cultured in Australia from 1994 to 1996. Similarly affected *P. monodon* from the Philippines were also infected with luminous vibriosis (*Vibrio harveyi*).

C.7.3 **Screening Methods**

More detailed information on methods for screening SMVD can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or at selected references.

There are no standard screening methods available for asymptomatic animals.

C.7.4 **Diagnostic Methods**

More detailed information on methods for diagnosis of SMVD can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000a), at http://www.oie.int, or at selected references.

C.7.4.1 *Presumptive*

C.7.4.1.1 Gross Observations (Level 1)

There are no specific clinical signs for SMVD. Juvenile *P. monodon* in grow-out ponds may show discolouration, lethargy, fouling and anorexia. Since this may be caused by several viral or bacterial infections, however, other diagnostic methods are required.

C.7.4.1.2 Cytology/Histopathology (Level II)

The histopathology associated with SMVD is not disease specific. In naturally infected juvenile *P. monodon,* haemocyte infiltration and cytolysis is focussed around the enteric epithelial surfaces. Experimental infections, using tissue extracts from shrimp with SMVD develop systemic infections manifest by systemic haemocytic infiltration, necrosis and sloughing of epithelial cells of the midgut and hepatopancreas.

C.7.4.2 *Confirmatory*

C.7.4.2.1 Transmission Electron Microscopy (TEM) (Level III)

SMV virions are found in the gut epithelial tissues. The viral particles measure approximately 20-25 *nm* in diameter and have hexagonal

[4] This disease is listed in the current FAO/NACA/OIE Quarterly Aquatic Animal Disease Reporting System as Spawner mortality syndrome ('Midcrop mortality syndrome').

(icosahedral) symmetry.

C.7.5 **Modes of Transmission**

Moribund and dead individuals are cannibalised by surviving animals, which is assumed to facilitate horizontal transmission.

C.7.6 **Control Measures**

Prevention of introduction of shrimp from SMV infected stock into historically uninfected areas is recommended. Daily removal of moribund animals from ponds, particularly early in production, has also been recommended. Stocking of ponds with progeny of spawners with SMV-negative faecal testing using PCR-probes has been shown to reduce mortality by 23%.

C.7.7 **Selected References**

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C.8 TAURA SYNDROME (TS)[5]

C.8.1 **Background Information**

C.8.1.1 *Causative Agent*

Taura Syndrome (TS) is caused by a virus, Taura Syndrome Virus (TSV) tentatively classified as a member of the *Picornaviridae* based on its morphology (31- 32 *nm* non-enveloped icosahedron), cytoplasmic replication, buoyant density of 1.338 g/ml, genome consisting of a linear, positive-sense ssRNA of approximately 10.2 kb in length, and a capsid comprised of three major (55, 40, and 24 kD) and one minor (58 kD) polypeptides. More detailed information about the pathogen can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (2000) and Lightner (1996).

C.8.1.2 *Host Range*

TSV infects a number of American penaeid species. The most susceptible species is the Pacific white shrimp *Penaeus vannamei,* although *P. stylirostris*, and *P. setiferus* can also be infected. Post-larvae and juvenile *P. schmittii*, *P. aztecus*, *P. duorarum, P. chinensis, P. monodon,* and *Marsupenaeus (Penaeus) japonicus* have been infected experimentally.

C.8.1.3 *Geographic Distribution*

Taura Syndrome was first detected in shrimp farms near the Taura River, Ecuador (hence the name of the disease) in 1992. It then spread throughout most shrimp growing regions of Latin America including Hawaii (infections successfully eradicated) and the Pacific coasts of Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama, and Peru.

TSV has also been reported from cultured shrimp along the Atlantic coasts of Belize, Brazil, Columbia, Mexico, and Venezuela and the southeastern U.S. states of Florida, South Carolina and Texas. TSV has, however, been successfully eradicated from cultured stocks in Florida and Belize. TSV is found in wild penaeids in Ecuador, El Salvador, Honduras, and Mexico. The only record of TSV in the eastern hemisphere is from Taiwan, Province of China, where the disease was likely introduced with *P. vannamei* from Central America.

C.8.2 **Clinical Aspects**

Taura Syndrome is particularly devastating to post-larval *P. vannamei* within approximately 14 to 40 days of stocking into grow-out ponds or tanks, however, larger stages may also be severely affected. Three distinct phases characterize TS disease progression: i) the acute stage, during which most mortalities occur; ii) a brief transition phase, and iii) a chronic 'carrier' stage. In the acute phase, the cuticular epithelium is the most severely affected tissue. In the chronic phase, the lymphoid organ becomes the predominant site of infection. In *P. vannamei*, the acute phase of infection may result in high mortalities (40-90%), while most strains of *P. stylirostris* appear resistant to fatal levels of infection. Survivors of acute TSV infection pass through a brief transition phase and enter the chronic phase which may persist for the rest of their lives. This sub-clinical phase of infection is believed to have contributed to the spread of the disease via carriage of viable TSV.

C.8.3 **Screening Methods**

Detailed information on methods for screening TSV can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000), at http://www.oie.int, or at selected references.

C.8.3.1 *Presumptive*

C.8.3.1.1 Gross Observation (Level I)

Any *Penaeus vannamei*, or other susceptible penaeid survivors of a TS outbreak, should be considered suspect carriers of TSV. However, there are no gross observation or Level I signs that can be used to screen sub-clinical carriers.

C.8.3.1.2 Histopathology (Level II)

Post-larvae, juveniles and adults can be screened using routine histological techniques and stains. Chronic stages of infection are characterised by the presence of spherical accumulations of cells in the lymphoid organ, referred to as 'lymphoid organ spheroids' (LOS). These masses are composed of presumed phagocytic hemocytes, which have sequestered TSV and aggregate within intertubular spaces of the lymphoid organs.

[5] Taura Syndrome (TS) is now classified as an OIE Notifiable Disease (OIE 2000).

C.8 Taura Syndrome (TS)

C.8.3.1.3 Immunoassays (Level III)

A commercial dot blot detection kit is available for TSV from DiagXotics (Wilton, CT, USA). ELISA kits using a TSV MAb have also been produced. These can be used to screen possible TSV carriers, but any positive results should be cross-checked with another confirmatory technique, or by bioassay, since visualisation of clinical signs or the virus is not possible with molecular screening techniques (this also applies to screening with PCR probes - C.8.3.1.5)

C.8.3.1.4 *In situ* Hybridization (Level III)

A commercial *in situ* hybridization detection kit is available for TSV from DiagXotics (Wilton, CT, USA). This technique is usually reserved for confirmation of observations made using routine histology (C.8.3.1.2), rather than as a standalone technique for screening.

C.8.3.1.5 PCR Probes (Level III)

An RT-PCR based assay uses shrimp haemolymph for screening purposes, giving the advantage of being able to screen live broodstock and assist selection of TSV-negative shrimp for spawning. Positive results from survivors of previous TSV outbreaks can be considered confirmatory, however, first time positive results from non-susceptible species or non-enzootic sources should be analyzed using another, confirmatory, technique for the same reasons given for dot-bot hybridization (C.8.3.1.3).

C.8.4 **Diagnostic Methods**

Detailed information on methods for diagnosis of TSV can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000), at http://www.oie.int, or at selected references.

C.8.4.1 *Presumptive*

C.8.4.1.1 Gross Observations (Level I)

Penaeus vannamei post-larvae or older shrimp may show a pale reddish discolouration, especially of the tail fan (Fig.C.8.4.1.1a,b) and pleiopods (hence the name "red tail" disease, applied by farmers when the disease first appeared in Ecuador). This colour change is due to expansion of the red chromatophores within the cuticular epithelium. Magnification of the edges of the pleiopods or uropods may reveal evidence of focal necrosis. Shrimp showing these signs typically have soft shells, an empty gut and often die during moulting. During severe epizootics, sea birds (gulls, terns, cormorants, *etc.*) may be attracted to ponds containing shrimp over 1 gm in size.

Although the transition stage of TS only lasts a few days, some shrimp may show signs of random, multi-focal, irregularly shaped melanized cuticular lesions (Fig.C.8.4.1.1c,d,e). These correspond to blood cell repair activity around the necrotic lesions induced by TSV infection of the cuticular epithelium. Such shrimp may, or may not, have soft cuticles and red discolouration, and may be behaving and feeding normally.

C.8.4.1.2 Histopathology (Level II)

Diagnosis of TS in acute stages of the disease requires histological (H&E stain preparations) demonstration of multi-focal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills (Fig.C.8.4.1.2a), hind-gut, esophagus and stomach (Fig.C.8.4.1.2b). Sub-cuticular connective tissue and striated muscle fibers basal or adjacent to affected cuticular epithelium may also show signs of necrosis. Rarely, the antennal gland tubule epithelium is affected. Cuticular lesions may contain foci of cells with abnormally eosinophilic (pink-staining) cytoplasm and pyknotic (condensed nucleoplasm) or karyorrhectic (fragmented nucleoplasm) nuclei. Remnants of necrotic cells are often abundant within acute phase lesions and appear as roughly spherical bodies (1-20 µm diameter) that range in stain uptake from eosinophilic to lightly basophilic (blue-staining). Another feature of acute TS is the absence of haemocyte infiltration, or other signs of a host defense response. These features combine to give acute phase TS lesions a "peppered" appearance (Fig.C.8.4.1.2c), that is considered to be diagnostic for the disease, and can be considered confirmatory (C.8.4.2.2) in susceptible species in enzootic waters. Confirmation by another technique is recommended for first time observations of these histopathological features, or their appearance in abnormal penaeid species or locations.

In the transitional phase of TS, the number and severity of the cuticular lesions that characterize acute phase infections decrease and affected tissues become infiltrated by haemocytes. These may become melanized

(C.8.4.1.1). If the acute cuticular lesions perforate the epicuticle, the affected surfaces may show evidence of colonization and invasion by *Vibrio* spp, or other secondary infections.

In the chronic phase of TS, the only sign of infection is the presence of prominent lymphoid organ spheres (LOS) (Fig.C.8.4.1.2d), which correspond to aggregations of presumed hemocytes within the intertubular spaces of the lymphoid organ.

(DV Lightner)

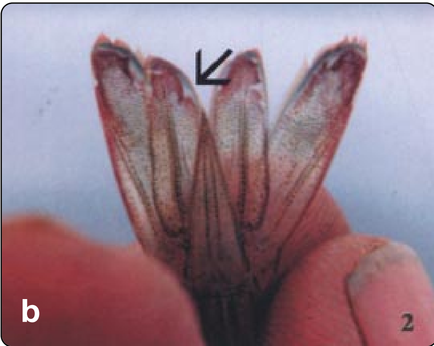

Fig. C.8.4.1.1a,b. a. Moribund, juvenile, pond-reared *Penaeus vannamei* from Ecuador in the peracute phase of Taura Syndrome (TS). Shrimp are lethargic, have soft shells and a distinct red tail fan; b. Higher magnification of tail fan showing reddish discoloration and rough edges of the cuticular epithelium in the uropods suggestive of focal necrosis at the epithelium of those sites (arrows).

(DV Lightner/F Jimenez)

Fig. C.8.4.1.1c,d,e. Juvenile, pond-reared *P. vannamei* (c – from Ecuador; d – from Texas; e – from Mexico) showing melanized foci mark sites of resolving cuticular epithelium necrosis due to TSV infection.

(DV Lightner)

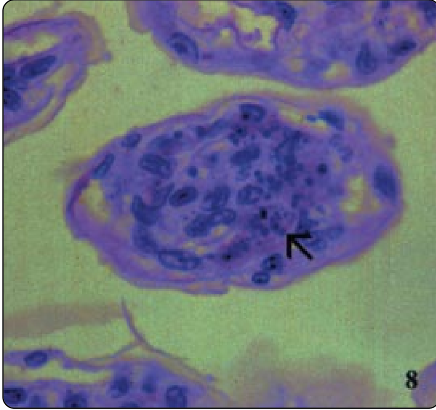

Fig. C.8.4.1.2a. Focal TSV lesions in the gills (arrow). Nuclear pykinosis and karyorrhexis, increased cytoplasmic eosinophilia, and an abundance of variably staining generally spherical cytoplasmic inclusions are distinguishing characteristics of the lesions. 900x magnification.

(DV Lightner)

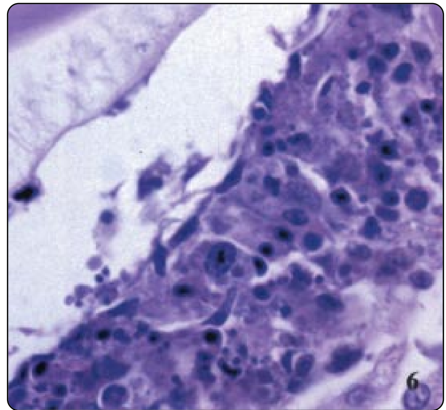

Fig. C.8.4.1.2c. Higher magnification of Fig. C.8.4.1.2b showing the cytoplasmic inclusions with pyknotic and karyorrhectic nuclei giving a 'peppered' appearance. Mayer-Bennett H&E. 900x magnification.

(DV Lightner)

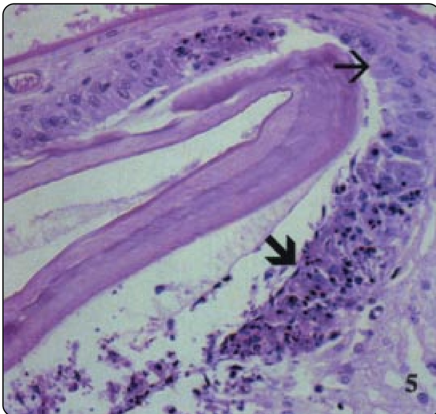

Fig. C.8.4.1.2b. Histological section through stomach of juvenile *P. vannamei* showing prominent areas of necrosis in the cuticular epithelium (large arrow). Adjacent to focal lesions are normal appearing epithelial cells (small arrows). Mayer-Bennett H&E. 300x magnification.

(DV Lightner)

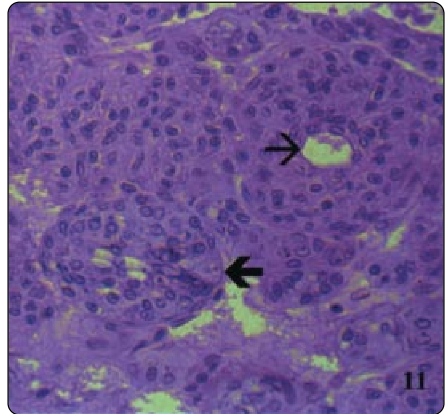

Fig. C.8.4.1.2d. Mid-sagittal section of the lymphoid organ (LO) of an experimentally infected juvenile *P. vannamei*. Interspersed among normal appearing lymphoid organ (LO) cords or tissue, which is characterized by multiple layers of sheath cells around a central hemolymph vessel (small arrow), are accumulations of disorganized LO cells that form LO 'spheroids". Lymphoid organs spheres (LOS) lack a central vessel and consists of cells which show karyomegaly and large prominent cytoplasmic vacuoles and other cytoplasmic inclusions (large arrow). Mayer-Bennett H&E. 300x magnification.

C.8.4.2 *Confirmatory*

C.8.4.2.1 Bioassay (Levels I/II)

Specific Pathogen Free (SPF) juvenile *Penaeus vannamei* can be used to test suspect TSV-infected shrimp. Three exposure methods can be used:

i) Suspect shrimp can be chopped up and fed to SPF juvenile *P. vannamei* held in small tanks. Another tank should hold SPF shrimp from the same source, but fed regular feed only (controls). If the suspect shrimp were positive for TSV, gross signs and histopathological lesions should become evident within 3-4 days of initial exposure. Significant mortalities usually occur by 3-8 days post-exposure. The control shrimp should stay healthy and show no gross or histological signs of TS.

ii) Whole shrimp collected from a presumptive TSV epizootic can be homogenized for inoculation challenge. Alternatively, heads may be used where presumptive TS signs appear to be at the transitional phase of development (melanized lesions) or where there are no clinical signs of infection (presumptive chronic phase) since this contains the lymphoid organ.

iii) Haemolymph samples may be taken from broodstock and used to expose SPF indicator shrimp, as for method ii) above.

C.8.4.2.2 Histopathology (Level II)

Observation of the lesions described under C.8.4.1.2 can be considered confirmatory for susceptible species from sources known to be enzootic for TSV.

C.8.4.2.3 Transmission Electron Microscopy (TEM) (Level III)

Transmission electron microscopy of acute phase epithelial lesions or lymphoid organ spheroids that demonstrate the presence of non-enveloped icosahedral viral particles, 31-32 *nm* in diameter, in the cytoplasm of affected cells, can be considered confirmatory where consistent with gross and histological clinical signs in a susceptible penaeid species. Further confirmation using molecular techniques (C.8.4.2.4-6) are recommended, however, for first-time diagnoses or detection in species other than those listed as being naturally or experimentally susceptible.

C.8.4.2.4 Dot Blot (Level III)

As described under C.8.3.1.3.

C.8.4.2.5 *In situ* Hybridization (Level III)

As described under C.8.3.1.4.

C.8.4.2.6 PCR Probes (Level III)

As described under C.8.3.1.5.

C.8.5 **Modes of Transmission**

Shrimps that have survived the acute and transitional phases of TS can maintain chronic sub-clinical infections within the lymphoid organ, for the remainder of their lives. These shrimp may transmit the virus horizontally to other susceptible shrimp. Vertical transmission is suspected, but this has yet to be conclusively demonstrated.

In addition to movement of sub-clinical carriers of TSV, aquatic insects and sea birds have been implicated in transmission of the disease. The water boatman, *Trichocorixa reticulata* (*Corixidae*), feeds on dead shrimp and is believed to spread TSV by flying from pond to pond. Laughing gull, *Larus atricilla,* faeces collected from around TSV-infected ponds in Texas during the 1995 epizootic, were also found to contain viable TSV. Viable TSV has also been found in frozen shrimp products.

C.8.6 **Control Measures**

In much of Central America where TS is enzootic, shrimp farm management has shifted towards increased use of wild caught *P. vannamei* PL, rather than hatchery-reared PL. This has improved survival to harvest. It is suspected that wild PL may have increased tolerance of TS due to natural exposure and selection of survivors. Another management strategy has been doubling post-larval stocking densities in semi-intensive pond culture. Heavy losses due to TS early in the production cycle are compensated for by the survivors (5-40% of the original number stocked) being TS tolerant. Selective breeding is showing promise for development of TSV resistant stocks of *P. vannamei* and *P. stylirostris* (which are resistant to both IHHNV and TSV). Initial results show a 20-40% improvement in survival.

Eradication depends on total removal of infected stocks, disinfection of the culture facility, avoidance of re-introduction of the virus

(from nearby culture facilities, wild shrimp, or sub-clinical carriers *etc*.), and re-stocking with TSV-free PL produced from TSV-free broodstock.

C.8.7 **Selected References**

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C.9 NUCLEAR POLYHEDROSIS BACULOVIROSES

(*BACULOVIRUS PENAEI* [BP] PvSNPV; MONODON BACULOVIRUS [MBV]PmSNPV)

C.9.1 **Background Information**

C.9.1.1 *Causative Agent*

Nuclear Polyhedrosis Baculoviroses (NPB) infections are caused by the Baculoviridae, *Baculovirus penaei* (BP - PvSNPV) and Mondon baculovirus (MBV – PmSNPV). The diseases associated with these viruses are Baculovirus disease, Nuclear polyhedrosis disease, polyhedral inclusion body virus disease (PIB), polyhedral occlusion body virus disease (POB) and *Baculovirus penaei* (BP) virus disease. More detailed information about the disease can be found at OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000).

C.9.1.2 *Host Range*

BP infects in a wide range of penaeid shrimp including *Penaeus duorarum*, *P. aztecus*, *P. setiferus*, *P. vannamei*, *P. stylirostris* and *P. marginatus*. BP has also been reported from *P. penicillatus, P. schmitti, P. paulensis* and *P. subtilis*.

MBV-type baculoviruses are, by definition, primarily found in cultured *P. monodon*. Other cocultured species may also acquire MBV-type virus infections, but these have not been associated with severe pathology, or developed nonmonodon reservoirs.

C.9.1.3 *Geographical Distribution*

BP is found throughout the Americas from the Gulf of Mexico to Central Brazil on the East Coast and from Peru to Mexico on the Pacific Coast. BP has also been found in wild shrimp in Hawaii. Multiple strains of BP are recorded within this geographic range.

MBV has been reported from Australia, East Africa, the Middle East, many Indo-Pacific countries and from south and eastern Asia. MBV-type viruses have also been found in sites associated with *P. monodon* culture in the Mediterranean and West Africa, Tahiti and Hawaii, as well as several locations in North and South America and the Caribbean.

C.9.2 **Clinical Aspects**

The impact of BP varies from species to species. *Penaeus aztecus* and *P. vannamei* are highly susceptible. *Penaeus stylirostris* is moderately susceptible and *P. monodon* and *P. setiferus* appear to be resistant/tolerant. In susceptible species, BP infection is characterised by a sudden onset of high morbidity and mortality in larval and post larval stages. Growth rates decrease, the shrimp stop feeding, appear lethargic and show signs of epibiont fouling (due to reduced grooming activity). The virus attacks the nuclei of hepatopancreas epithelia but can also infect mid-gut epithelia. Although infections may be chronic to acute, with high cumulative mortality, presence of the BP virus is not always associated with disease and post-larvae older than 63 days show no clinical signs of infection (see C.9.6).

MBV causes similar clinical signs to BP, due to similar infection of the hepatopancreatic and mid-gut epithelial nuclei. Infections of MBV may also occur in the lymphoid organ. Larval stages of *P. monodon* are particularly susceptible, however, prevalences of >45% may be present in juvenile and adult developmental stages with no overt clinical effects.

C.9.3 **Screening Methods**

More detailed information on methods for screening NPB can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000), at http://www.oie.int, or at selected references.

C.9.3.1 *Presumptive*

There are no presumptive screening methods for asymptomatic carriers of BP and MBV, since direct microscopic methods (C.9.3.2) demonstrating the characteristic occlusion bodies (tetrahedral for BP and spherical-ovoid for MBV) are considered to be confirmatory.

C.9.3.2 *Confirmatory*

C.9.3.2.1 Wet Mount of Fresh Tissue (Level I/II)

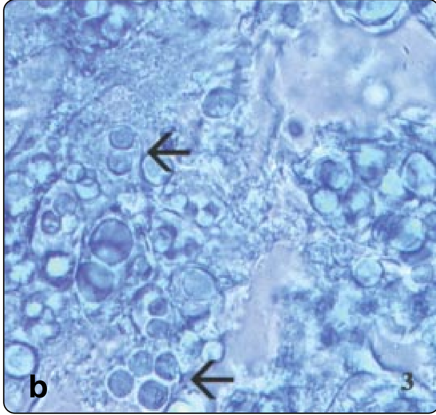
BP infections can be confirmed by bright-field or phase contrast microscopic observation of single or multiple tetrahedral (polyhedral) inclusion (occlusion) bodies (Fig. C.9.3.2.1a) within enlarged nuclei of hepatopancreas or midgut epithelia. These bodies can range in size from 0.1-20.0 μm (modal range = 8-10 μm) along the perpendicular axis from the base of the pyrimidal shape to the opposite point.

MBV infections observed using the same microscope apparatus appear as single or multiple spherical or sub-spherical inclusion bodies within enlarged nuclei of hepatopancreas

C.9 Nuclear Polyhedrosis Baculoviroses

(*Baculovirus penaei* [BP] PvSNPV; Monodon Baculovirus [MBV]PmSNPV)

(DV Lightner)



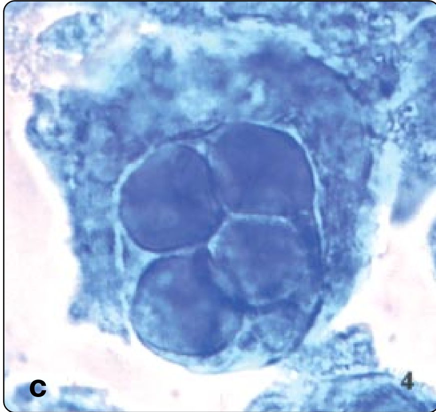
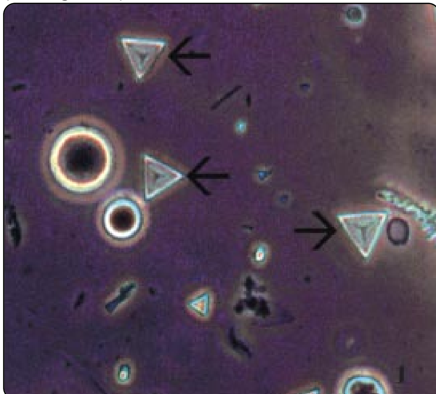
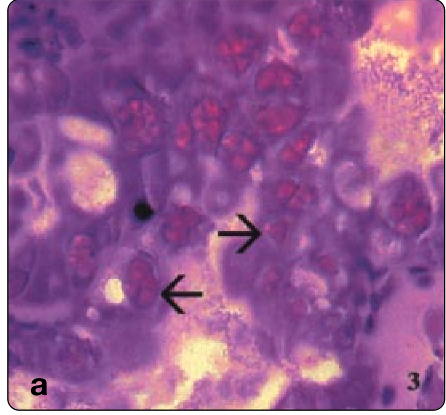


Fig. C.9.3.2.1b,c. Mid and high magnification views of tissue squash preparations of the hepatopancreas (HP) from PL of *P. monodon* with MBV infections. Most HP cells in both PLs usually display multiple, generally spherical, intranuclear occlusion bodies (arrow) that are diagnostic for MBV. 0.1% malachite green. 700x (b) and 1 700x (c) magnifications.

(DV Lightner)



(DV Lightner)



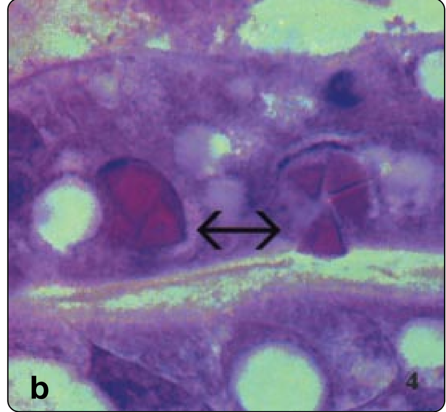


Fig. C.9.3.2.3a,b. a. Mid-magnification view of mid-sagittal sections of PL of *P. vannamei* with severe BP infections of the hepatopancreas showing multiple eosinophilic BP tetrahedral occlusion bodies within markedly hypertrophied hepatopnacreas (HP) cell nuclei (arrows). Mayer-Bennett H&E. 700x magnification; b. High magnification of an HP tubule showing several BP-infected cells that illustrate well the intranuclear, eosinophilic, tetrahedral occlusion bodies of BP (arrows). Mayer-Bennett H&E. 1800x magnification.

Fig. C.9.3.2.1a. Wet mount of feces from a *P. vannamei* infected with BP showing tetrahedral occlusion bodies (arrows) which are diagnostic for infection of shrimp's hepatopancreas or midgut epithelial cells. Phase contrast, no stain. 700x magnification.

or midgut epithelia. MBV occlusion bodies measure 0.1 –20.0 µm in diameter (Fig. C.9.3.2.1b,c). The occlusion bodies can be stained using a 0.05% aqueous solution of malachite green, which stains them more densely than surrounding, similarly sized spherical bodies (cell nuclei, secretory granules, lipid droplets, etc.).

C.9.3.2.2 Faecal Examination (Level I/II)

Make wet mounts of faecal strands and examine for occlusion bodies, as described for fresh tissue mounts (C.9.3.2.1).

C.9.3.2.3 Histopathology (Level II)

Tissues from live or moribund (but not dead, due to rapid liquefaction of the target organ – the hepatopancreas) shrimp should be fixed in Davidson's fixative to ensure optimum fixation of the hepatopancreas (10% buffered formalin provides sub-optimal hepatopancreas preservation). The fixative should be administered by direct injection into the hepatopancreas. The cuticle should be cut along the dorsal line of the cephalothorax to enhance fixative penetration of the underlying tissues and the tissues should be fixed for 24-48 hr before transfer to 70% ethanol for storage. The tissues can then be processed for routine paraffin embedding, sectioning at 5-7 µm thickness and staining with Harris' haematoxylin and eosin or other Giemsa or Gram tissue-staining methods. Brown and Brenn's histological Gram stain provides intense red or purple colouration of both MBV (see also Fig. C.5.4.2.1d – C.5) and BP occlusion bodies (Fig. C.9.3.2.3a,b), aiding in their differentiation from surrounding tissues.

C.9.3.2.4 Polymerase Chain Reaction Assays (Level III)

Two primers sequences are available for the MBV polyhedrin gene (Lu *et al* 1993) and another pair are available for a 1017bp fragment of the viral genome (Mari *et al* 1993). Details on the PCR procedures for screening tissue or faecal samples are provided in the OIE Diagnostic Manual (OIE 2000) or selected references (C.9.7).

C.9.4 **Diagnostic Methods**

More detailed information on methods for diagnosis of NPB can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000), at http://www.oie.int, or at selected references.

C.9.4.1 *Presumptive*

C.9.4.1.1 Gross Observations (Level I)

Gross signs of BP vary between susceptible species but include decreased growth, cessation of feeding and preening, lethargy and increased epibiont fouling. Some shrimp may exhibit a white mid-gut line through the ventral abdominal cuticle. None of these symptoms are specific to BP, but can be considered suspect in susceptible species and at early developmental/post-larval stages which have a history of being affected by BP.

MBV causes similar clinical signs to BP, but principally affects larval of *P. monodon* with an inverse correlation between larval age and pathogenic effects. Adults can be infected with no overt signs (see C.9.3). As with BP, these signs are not specific to MBV.

C.9.4.2 *Confirmatory*

C.9.4.2.1 Wet Mount of Fresh Tissue (Level I/II)

As described for C.9.3.2.1.

C.9.4.2.2 Faecal Examination (Level I/II)

As described for C.9.3.2.2.

C.9.4.2.3 Histopathology (Level II)

As described for C.9.3.2.3.

C.9.4.2.4 Autofluorescence with phloxine stain (Level II)

An aqueous solution of 0.001% phloxine used on tissue squash preparations or faeces, will cause occlusion bodies of both BP and MBV to fluoresce yellow-green when examined using a fluorescent microscope (barrier filter 0-515 *nm* and exciter filter of 490 *nm*) (Thurman *et al.* 1990). The same effect is achieved using 0.005% phloxine in routine haematoxylin and eosin stain of histological tissue preparations.

C.9.4.2.5 Transmission Electron Microscopy (TEM) (Level III)

BP virions are rod-shaped with an enveloped nucleocapsid measuring 286-337 nm x 56-79 nm. The virions are found either free or occluded within a crystalline protein matrix (the occlusion body). In early infections, virions are found

in association with nuclear enlargements, aberrant stromatic patterns of the nucleoplasm, degenerate nucleoli, and nuclear membrane proliferation into labyrinths. Occlusion bodies occur during later stages of infection.

MBV has been shown to have two types of occlusion bodies using electron microscopic examinations (Ramasamy *et al.* 2000). Type 1 has a paracrystalline array of polyhedrin units within a lattice work spacing of 5-7 *nm*, which contains occluded virions (along with a few peripheral non-occluded virions) that have a double envelope and measure 267 ± 2 x 78 ± 3 nm. Type 2 occlusion bodies consist of non-crystalline, granulin-like sub-units 12 nm in diameter, containing mostly non-occluded virions measuring 326 ± 4 x 73 ± 1 nm. In addition, a non-enveloped stage has recently been detected (Vickers *et al.* 2000) in the cytoplasm of infected cells and close association with the nuclear membrane.

C.9.4.2.6 *In situ* Hybridization (Level III)

Details of the preparation and analytical procedures requried for *in situ* hybridisation for confirming BP and MBV infections are provided in the OIE Diagnostic Manual (OIE 2000a) under both the Nuclear Polyhedrosis Baculoviroses chapter (Chapter 4.2.2) as well as the Infectious Hypodermal and Haematopoietic Necrosis chapter (Chapter 4.2.3).

C.9.5 **Modes of Transmission**

BP and MBV are both transmitted orally via uptake of virus shed with the faeces of infected shrimp (C.9.3.2.2), or cannibalism on dead and dying shrimp. Infected adults have also been shown to infect their offspring via faecal contamination of the spawned egg masses.

C.9.6 **Control Measures**

Overcrowding, chemical and environmentally induced stress, have all been shown to increase the virulence of MBV and BP infections in susceptible shrimp species under culture conditions.

Exposure of stocks to infection can be avoided by pre-screening the faeces of potential broodstock and selecting adults shown to be free of faecal contamination by occlusion bodies of either baculovirus. Prevention of infections may also be achieved by surface disinfection of nauplii larvae or fertilised eggs with formalin, iodophore and filtered clean seawater as follows:

- Collect nauplii and wash in gently running sea water for 1-2 minutes.
- Immerse the nauplii in a 400 ppm solution of formalin for 1 minute followed by a solution of 0.1 ppm iodine for an additional minute. The same procedure can be used on fertilised eggs except the formalin concentration is reduced to 100ppm.
- Rinse the treated nauplii in running sea water for 3-5 min and introduce to the hatchery.

Eradication of clinical outbreaks of BP and MBV may be possible in certain aquaculture situations by removal and sterile disposal of infected stocks, disinfection of the culture facility, the avoidance of re-introduction of the virus (from other nearby culture facilities, wild shrimp, *etc*.).

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BACTERIAL DISEASE OF SHRIMP
C.10 NECROTISING HEPATOPANCREATITIS (NHP)

C.10.1 **Background Information**

C.10.1.1 *Causative Agent*

Necrotising Hepatopancreatitis (NHP) is caused by a bacterium that is relatively small, highly pleomorphic, Gram negative, and an apparent obligate intracellular pathogen. The NHP bacterium has two morphologically different forms: one is a small pleomorphic rod and lacks flagella; while the other is a longer helical rod possessing eight flagella on the basal apex of the bacterrium, and an additional flagellum (or possibly two) on the crest of the helix.. The NHP bacterium occupies a new genus in the alpha Proteobacteria, and is closely related to other bacterial endosymbionts of protozoans. NHP is also known as Texas necrotizing hepatopancreatitis (TNHP), Texas Pond Mortality Syndrome (TPMS) and Peru necrotizing hepatopancreatitis (PNHP). More information about the disease is found in Lightner (1996).

C.10.1.2 *Host Range*

NHP can infect both *Penaeus vannamei* and *P. stylirostris* but causes higher mortalities in the former species. NHP has also been reported in *P. aztecus*, *P. californiensis* and *P. setiferus.*

C.10.1.3 *Geographic Distribution*

NHP was first described in Texas in 1985. Other outbreaks have been reported in most Latin American countries on both the Pacific and Atlantic Ocean coasts, including Brazil, Costa Rica, Ecuador, Mexico, Panama, Peru and Venezuela.

C.10.2 **Clinical Aspects**

The NHP bacterium apparently infects only the epithelial cells lining the hepatopancreatic tubules, and, to date, no other cell type has been shown to become infected. The hepatopancreas in shrimp is a critical organ involved in food digestion, nutrient absorption and storage, and any infection has obvious and serious consequences for the affected animal, from reduced growth to death. Various environmental factors appear to be important for the onset of NHP clinical signs; the most prominent ones are water salinity over 16 ppt (parts per thousand) and water temperature of 26˚C or higher.

C.10.3 **Screening Methods**

C.10.3.1 *Confirmatory*

C.10.3.1.1 Dot Blot for Asymptomatic Animals (Level III)

A commercial dot blot detection kit is available for NHP from DiagXotics (Wilton, CT, USA).

C.10.3.1.2 *In situ* Hybridization (Level III)

A commercial *in situ* hybridization detection kit is available for NHP from DiagXotics (Wilton, CT, USA).

C.10.3.1.3 Polymerase Chain Reaction (PCR) (Level III)

Samples of hepatopancreas are fixed in 70% ethanol and triturated prior to processing. DNA is isolated as follows: 25 mg of the triturated hepatopancreas is suspended in 250 µl of digestion buffer (50 mM Tris, 20 mM EDTA, 0.5% SDS, pH 8.5) in 0.5 ml eppendorf tubes. Proteinase K (7.5 µl of a 20 mg ml^{-1} stock solution) is added and the tube incubated at 60°C for 2 h with periodic vortexing. The tube is then incubated at 95°C for 10 min to inactivate the proteinase K. The tube is then centrifuged for 3 min at 13,000 rpm (16,000 x *g*) and 75 µl of the supernatant applied to a CHROMA SPIN TE-100 (Clontech Labs) column and centrifuged in a horizontal rotor according to the manufacturer's protocol. The solution collected by centrifugation is diluted 1:100 and 1:1000 in distilled water prior to use in the PCR assay.

Below is the sequence of oligonucleotide primers used for amplifying variable regions of the 16S rRNA sequence:

Forward: 5'-ACG TTG GAG GTT CGT CCT TCA G-3'
Reverse1 5'-TCA CCC CCT TGC TTC TCA TTG T-3'
Reverse2 5'-CCA GTC ATC ACC TTT TCT GTG GTC-3'

The forward primer and reverse primer 1 amplify a 441 bp fragment, the forward primer and reverse primer 2 amplify a 660 bp fragent. PCR is performed in 50 µl reactions containing 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM MgCl, 200 mM deoxynucleotides, 0.5 m?M of the forward and the paired reverse primers and 0.03 to 0.3 µg of template DNA. The reactants are heated to 94°C in a programmable thermocycler

C.10 Necrotising Hepatopancreatitis (NHP)

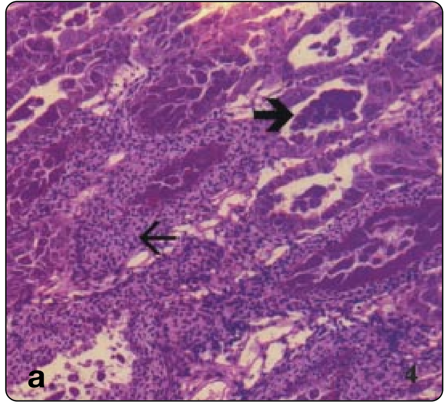
(DV Lightner)

Fig. C.10.4.1.1. Juvenile *P. vannamei* with NHP showing markedly atrophied hepatopancreas, reduced to about 50% of its normal volume.

(DV Lightner)

Fig. C.10.4.1.2. Wet- mount of the HP of infected shrimp with inflamed hemocyte, melanized HP tubules and absence of lipid droplets. No stain. 150x magnification.

(DV Lightner)



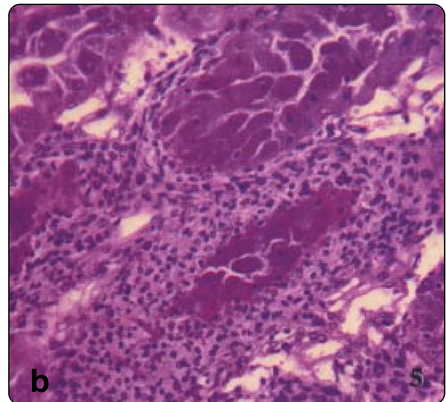


Fig. C.10.4.1.3a,b. Low and mid-magnification of photographs of the HP of a severely NHP infected juvenile *P. vannamei.* Severe hemocytic inflammation of the intratubular spaces (small arrow) in response to necrosis, cytolysis and sloughing of HP tubule epithelial cells (large arrow), are among the principal histopathological changes due to NHP. Mayer-Bennett H&E. 150x (a) and 300x (b) magnifications.

(DV Lightner)

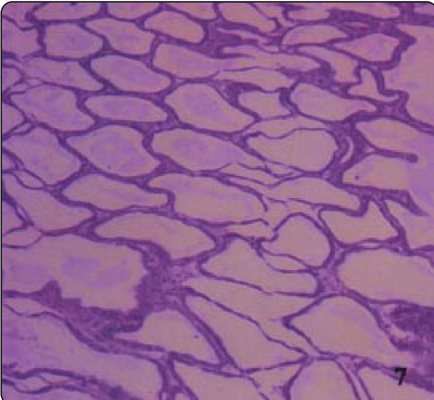


Fig. C.10.4.1.3c. Low magnification view of the HP of a juvenile *P.vannamei* with severe, chronic NHP. The HP tubule epithelium is markedly atrophied, resulting in the formation of large edematous (fluid filled or "watery areas in the HP. Mayer-Bannett H & E. 100x magnification.

C.10 Necrotising Hepatopancreatitis (NHP)

prior to adding 1.25 U of Amplitaq DNA polymerase. The final solution is then overlayed with mineral oil. The amplification profile consists of 35 cycles of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C with an additional 5 min at 72°C following the final cycle. PCR products is examined by electrophoresis in 1% agarose in TAE buffer containing 0.5 m?g ml^{-1} ethidium bromide.

C.10.4 **Diagnostic Methods**

More detailed information on methods for diagnosis of NHP can be found in Lightner (1996) or in selected references.

C.10.4.1 *Presumptive*

C.10.4.1.1 Gross Observations (Level 1)

A wide range of gross signs can be used to indicate the possible presence of NHP. These include: lethargy, reduced food intake, higher food conversion ratios, anorexia and empty guts, noticeable reduced growth and poor length weight ratios ("thin tails"); soft shells and flaccid bodies; black or darkened gills; heavy surface fouling by epicommensal organisms; bacterial shell disease, including ulcerative cuticle lesions or melanized appendage erosion; and expanded chromatophores resulting in the appearance of darkened edges in uropods and pleopods. The hepatopancreas may be atrophied (Fig.C.10.4.1.1) and have any of the following characteristics: soft and watery; fluid filled center; paled with black stripes (melanized tubules); pale center instead of the normal tan to orange coloration. Elevated mortality rates reaching over 90% can occur within 30 days of onset of clinical signs if not treated.

C.10.4.1.2 Wet Mounts (Level II)

Wet mounts of the hepatopancreas of shrimp with NHP may show reduced or absent lipid droplets and/or melanized hepatopancreas tubules (Fig.C.10.4.1.2).

C.10.4.1.3 Histopathology (Level II)

NHP is characterised by an atrophied hepatopancreas showing moderate to extreme atrophy of the tubule mucosa and the presence of the bacterial forms through histological preparations. Principal histopathological changes due to NHP include hemocytic inflammation of the intertubular spaces in response to necrosis, cytolysis, and sloughing of hepatopancreas tubule epithelial cells (Fig. C.10.4.1.3a,b). The hepatopancreas tubule epithelium is markedly atropied, resulting in the formation of large edematous (fluid filled or "watery") areas in the hepatopancreas (Fig.C.10.4.1.3c).Tubule epithelial cells within granulomatous lesions are typically atrophied and reduced from simple columnar to cuboidal in morphology. They contain little or no stored lipid vacuoles (Fig.C.10.4.1.3d) and markedly reduced or no secretory vacuoles.

C.10.4.2 *Confirmatory*

C.10.4.2.1 Transmission Electron Microscopy (TEM) (Level III)

Two distinct versions of the NHP bacterium occur in infected hepatopancreatic cells. The first is a rod-shaped rickettsial-like form measuring 0.3 µm x 9 µm which lacks flagella. The second is a helical form (Fig.C.10.4.2.1) measuring 0.2 µm x 2.6-2.9 µm which has eight periplasmic flagella at the basal apex of the bacterium and an additional 1-2 flagella on the crest of the helix.

C.10.4.2.2 Dot Blot for Asymptomatic Animals (Level III)

A commercial dot blot detection kit is available for NHP from DiagXotics (Wilton, CT, USA).

C.10.4.2.3 *In situ* Hybridization (Level III)

A commercial *in situ* hybridization detection kit is available for NHP from DiagXotics (Wilton, CT, USA).

C.10.4.2.4 Polymerase Chain Reaction (PCR) (Level III)

As described for C.10.3.1.3

C.10.5 **Modes of Transmission**

Early detection of clinical NHP is important for successful treatment because of the potential for cannibalism to amplify and transmit the disease. Molecular testing of PL from infected broodstock indicates that vertical transmission does not occur.

C.10.6 **Control Measures**

Periodic population sampling and examination (through histopathology, TEM or commercial gene probe) are highly recommended in farms

(DV Lightner)

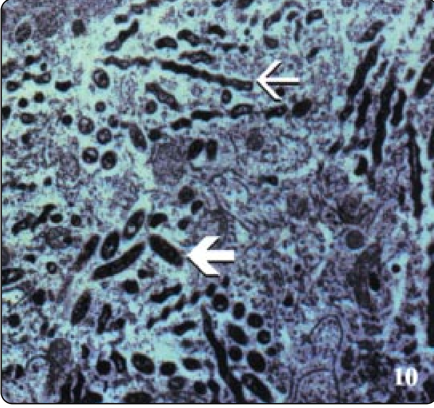


Fig. C.10.4.1.3d. The HP tubule epithelial cells show no cytoplasmic lipid droplets, but instead contain masses of the tiny, non-membrane bound, intracytoplasmic NHP bacteria (arrow). Mayer-Bennett H&E. 1700x magnification.

(DV Lightner)

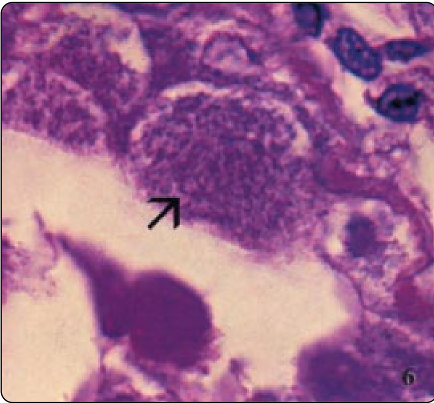


Fig. C.10.4.2.1. Low magnification TEM of a hepatopancreatocyte from a juvenile *P. vannamei* with NHP. Profiles of intracellular rod-shaped forms (large arrow) and helical forms (small arrow) of the NHP bacterium are abundant in the cytoplasm. 10 000x magnification.

with a history of NHP occurrence and where environmental conditions favor outbreaks. The use of the antibiotic oxytetracycline (OTC) in medicated feeds is probably the best NHP treatment currently available, particularly if **disease** presence is detected early.

There is also some evidence that deeper production ponds (2 m) and the use of hydrated lime ($Ca(OH)_2$) to treat pond bottoms during pond preparation before stocking can help reduce NHP incidence. Preventive measures can include raking, tilling and removing pond bottom sediments, prolonged sun drying of ponds and water distribution canals for several weeks, disinfection of fishing gear and other farm equipment using calcium hypochlorite and drying and extensive liming of ponds.

C.10.7 Selected References

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FUNGAL DISEASE OF CRAYFISH
C.11 CRAYFISH PLAGUE

C.11.1 **Background Information**

C.11.1.1 *Causative Agent*

Crayfish Plague (also known as Krebspest, Kraftpest, 'la peste' or 'crayfish aphanomyciasis') is caused by the Oomycete fungus, *Aphanomyces astaci*. This is a close relative of species associated with serious finfish diseases, such as *A. invadans*, in Epizootic Ulcerative Syndrome (EUS) of South-East Asia (see section F.11).

C.11.1.2 Host Range

Crayfish plague affects the Noble crayfish *Astacus astacus* of north-west Europe, the stone crayfish *Austropotamobius pallipes* of south-west and west Europe, the mountain crayfish *Austropotamobius torrentium* of south-west Europe, and the slender clawed or Turkish crayfish *Astacus leptodactylus* of eastern Europe and Asia Minor. The Chinese mitten crab *(Eriocheir sinensis)* can be infected experimentally. North American crayfish (*Pacifasticus leniusculus*, the signal crayfish, and *Procambarus clarkii*, the Louisiana swamp crayfish) can also be infected by *A. astaci*, but are relatively tolerant of the disease, only exhibiting clinical signs under intensive culture conditions.

C.11.1.3 *Geographical Distribution*

Aphanomyces astaci is widespread in Europe, as well as in North America. The disease first appeared in northern Italy in the mid 19th century, and then spread down to the Balkans and Black Sea, as well as into Russia, Finland and Sweden. In the 1960's the disease appeared in Spain with further spread to the British Isles, Turkey, Greece and Norway in the 1980's.

C.11.2 **Clinical Aspects**

The hyphae of *A. astaci* grow throughout the non-calcified parts of the cuticle and may extend along the nerve cord. The more disease tolerant species of crayfish (North American) encapsulate the fungal hyphae within melanised nodules, arresting the hyphal proliferation. Susceptible species appear incapable of producing such a defense reaction, and the fungus proliferates throughout the epicuticle and exocuticular layers of the exoskeleton. The cuticle and related soft-tissue damage leads to death which, under warm water conditions, can be rapid and result in 100% mortality. Resistant North American species that survive initial infection can become sub-clinical carriers of the fungus. Under adverse holding conditions, however, such infections may become pathogenic.

C.11.3 **Screening Methods**

More detailed information on methods for screening crayfish plague can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000), at http://www.oie.int or selected references.

C.11.3.1 *Presumptive*

C.11.3.1.1 Gross Observations (Level I)

Melanized spots in the cuticle of any crayfish species may be indicative of crayfish plague survival. Such crayfish should be considered to be potential carriers of the disease and screened for *Aphanomyces astaci* using confirmatory diagnostic techniques (C.11.3.2 and C.11.4.2).

C.11.3.1.2 Microscopy (Level I/II)

Foci of infection as described under C.12.3.1.1, may not be readily visible. Examination using a dissecting microscope may reveal small whitened patches in the muscle tissues underlying thin spots in the cuticle. There may also be brownish discolouration of the cuticle. Fine brown lines through the cuticle should also be considered as suspect fungal hyphae. The areas that should be examined closely are the intersternal soft-ventral cuticle of the abdomen and tail; the cuticle between the carapace and tail, the joints of the periopods (especially the proximal joints), the perianal cuticle and the gills.

C.11.3.2 *Confirmatory*

C.11.3.2.1 Culture (Level II)

The fungus can be isolated from suspect cuticle and tissues using an agar medium that contains yeast extract, glucose and antibiotics (penicillin G and oxolinic acide) made up with natural (not demineralised) river water. Identification to species requires morphological characterisation of the sexual reproductive parts of the fungus, however, these stages are absent in *A. astaci*, thus, confirmation of infection is usually based on isolation of fungal colonies with the following characteristics (since no other closely-related Oomycetes are known to infect crayfish):

C.11 Crayfish Plague

- growth within the agar medium (unless cultured at < 7°C, which promotes superficial growth);
- colourless colonies;
- aseptate, highly branching, vegetative hyphae, 7-9 µm in diameter (min-max 5-10 µm);
- young hyphae are densely packed with coarse, granular cytoplasm and contain highly refractile globules;
- older hyphae are highly vacuolated and the oldest hyphae appear to be empty

When thalli are transferred from the culture medium to sterile distilled water, they develop sporangia within 12-15 h (20°C) or 20-30 h (16°C). Elongate, irregularly amoeboid shaped spores are released and rapidly encyst as a mass around the sporangial tip (Fig.C.11.3.2.1a). Encysted primary spores measure 9-11 µm in diameter (min-max 8-15 µm). Release of the secondary zoospores occurs from papillae that develop on the surface of the primary spore cyst. This occurs at temperatures as low as 4°C, peaking at 20°C and stopping at temperatures >24°C. The zoospores have lateral flagella and measure 8 x 12 µm. More details on culture media, techniques and developmental stage morphology are provided in the OIE Manual (OIE 2000).

C.11.3.2.2 Bioassay (Level I/II)

Confirmation of crayfish plague can be done using zoospores cultured from fungal isolates from suspect crayfish tissues. Rapid mortalities in the susceptible crayfish, along with re-isolation of the fungus as described above, should be considered conclusive for *A. astaci*.

C.11.4 Diagnostic Methods

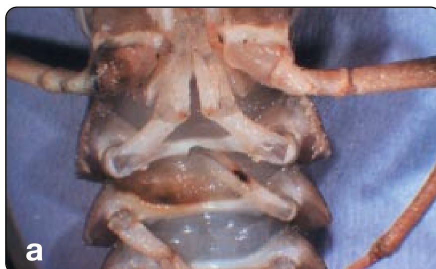
More detailed information on methods for diagnosis of crayfish plague can be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000), at http://www.oie.int or selected references.

There is no other disease, or pollution effect, that can cause total mortality of crayfish but leave all other animals in the same water unharmed. In such situations and with known susceptible species, presumptive diagnosis can be fairly conclusive. In first-time cases or in situations with resistant species, however, confirmatory isolation of the pathogen is recommended.

(EAFP/DJ Alderman)

Fig. C.11.3.2.1a. Fresh microscopic mount of a piece of infected exoskeleton showing fungal spores.

(EAFP/DJ Alderman)



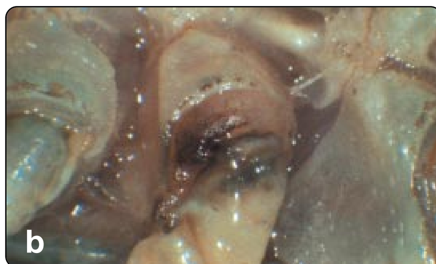


Fig. C.11.4.1.1a,b. Clinical signs of infected crayfish showing whitened necrotic musculature in the tail, and often accompanied in chronic infections by melanisation (blackening) of affected exoskeleton.

C.11.4.1 *Presumptive*

C.11.4.1.1 Gross observation (Level I)

Large numbers of crayfish showing activity during daylight should be considered suspect, since crayfish are normally nocturnal. Some may show uncoordinated movement, easily tip onto their backs, and be unable to right themselves.

Gross clinical signs of crayfish plague vary from none to a wide range of external lesions. White

patches of muscle tissue underlying transparent areas of cuticle (especially the ventral abdomen and periopod joints), and focal brown melanised spots (Fig.C.11.4.1.1a,b), are the most consistent signs.

C.11.4.1.2 Microscopy (Level I/II)

As for C.11.3.1.2.

C.11.4.2 *Confirmatory*

C.11.4.2.1 Culture (Level II)

As for C.12.3.2.1, diagnosis of crayfish plague requires the isolation and characterisation of the pathogen, *A. astaci,* using mycological media fortified with antibiotics to control bacterial contamination. Isolation is only likely to be successful before or within 12 hours of the death of infected crayfish.

C.11.4.2.2 Bioassay (Level I/II)

As for C.11.3.2.2.

C.11.5 **Mode of Transmission**

Transmission is horizontal and direct via the motile biflagellate zoospore stage of *A. astaci*, which posseses a positive chemotaxis towards crayfish. The disease can spread downstream at the speed of flow of the river, and has been documented to spread upstream at 2-4 km per year. The upstream spread is suspected to driven by movements of crayfish between infection and the terminal stages of the disease.

Transmission has also been linked to the water used to move fish between farms, as well as to contaminated equipment (boots, fishing gear, crayfish traps, etc.). Introductions of North American crayfish for crayfish farming are believed to have been the source of the European outbreaks of crayfish plague.

C.11.6 **Control Measures**

There is no treatment for crayfish plague, and the high levels of mortality have precluded natural selection for disease resistance in the most susceptible species (some populations are now endangered). Control of the disease is best achieved by preventing introductions or escape of crayfish into unaffected waters. In addition, movement of water or any equipment between affected to unaffected watersheds should be avoided or undertaken with disinfection precautions. Sodium hypochlorite and iodophores can be used to disinfect equipment and thorough drying (>24 hours) is also effective, since oomycetes cannot withstand desiccation.

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ANNEX C.AI. OIE REFERENCE LABORATORY FOR CRUSTACEAN DISEASES

Disease	Expert/Laboratory
Crustacean pathogens	**Prof. D. Lightner** Aquaculture Pathology Section Department of Veterinary Science University of Arizona Building 90, Room 202 Tucson AZ 85721 USA Tel: (1.520) 621.84.14 Fax: (1.520) 621.48.99 E-mail: dvl@u.arizona.edu
	Prof. S.N. Chen Department of Zoology Director, Institute of Fishery Biology National Taiwan University No. 1 Roosevelt Road Section 4 , Taipei, Taiwan 10764 TAIWAN PROVINCE of CHINA Tel: 886-2-368-71-01 Fax: 886-2-368-71-22 E-mail: snchen@cc.ntu.edu.tw

ANNEX C.AII. LIST OF REGIONAL RESOURCE EXPERTS FOR CRUSTACEAN DISEASES IN ASIA-PACIFIC[1]

Disease	Expert
Shrimp diseases	**Dr. Richard Callinan** NSW Fisheries, Regional Veterinary Laboratory Wollongbar NSW 2477 **AUSTRALIA** Tel (61) 2 6626 1294 Mob 0427492027 Fax (61) 2 6626 1276 E-mail: richard.callinan@agric.nsw.gov.au
	Dr. Indrani Karunasagar Department of Fishery Microbiology University of Agricultural Sciences Mangalore – 575 002 **INDIA** Tel: 91-824 436384 Fax: 91-824 436384 E-mail: mircen@giasbg01.vsnl.net.in
	Dr. C.V. Mohan Department of Aquaculture College of Fisheries University of Agricultural Sciences Mangalore-575002 **INDIA** Tel: 91 824 439256 (College); 434356 (Dept), 439412 (Res) Fax: 91 824 438366 E-mail: cv_mohan@yahoo.com
	Prof. Mohammed Shariff Faculty of Veterinary Medicine Universiti Putra Malaysia 43400 Serdang, Selangor **MALAYSIA** Tel: 603-9431064; 9488246 Fax: 603-9488246; 9430626 E-mail: shariff@vet.upm.edu.my
	Dr. Jie Huang Yellow Sea Fisheries Research Institute Chinese Academy of Fishery Sciences 106 Nanjing Road Qingdao, Shandong 266071 **PEOPLE'S REPUBLIC of CHINA** Tel: 86 (532) 582 3062 Fax: 86 (532) 581 1514 E-mail: aqudis@public.qd.sd.cn
	Dr. Jian-Guo He School of Life Sciences Zhongshan University Guangzhou 510275 **PEOPLE'S REPUBLIC of CHINA** Tel: +86-20-84110976 Fax: +86-20-84036215 E-mail: lsbrc05@zsu.edu.cn
	Dr. Juan D. Albaladejo Fish Health Section Bureau of Fisheries and Aquatic Resources Arcadia Building, 860 Quezon Avenue Quezon City, Metro Manila **PHILIPPINES**

	Tel/Fax: 632-372-5055 E-mail: jalbaladejo99@yahoo.com
	Dr. Joselito R. Somga Fish Health Section Bureau of Fisheries and Aquatic Resources Arcadia Building, 860 Quezon Avenue Quezon City, Metro Manila **PHILIPPINES** Tel/Fax: 632-372-5055 E-mail: jrsomga@edsamail.com.ph
	Dr. Leobert de la Pena Fish Health Section Aquaculture Department Southeast Asian Fisheries Development Center Tigbauan, Iloilo 5021 **PHILIPPINES** Tel: 63 33 335 1009 Fax: 63 33 335 1008 E-mail: leobert65@yahoo.com; leobertd@aqd.seafdec.org.ph
	Dr. P.P.G.S.N. Siriwardena Head, Inland Aquatic Resources and Aquaculture National Aquatic Resources Research and Develoment Agency Colombo 15, **SRI LANKA** Tel: 941-522005 Fax: 941-522932 E-mail: sunil_siriwardena@hotmail.com
	Dr. Yen-Ling Song Department of Zoology College of Science National Taiwan University 1, Sec. 4, Roosevelt Rd. **TAIWAN PROVINCE OF CHINA** E-mail: yenlingsong@hotmail.com
	Dr. Pornlerd Chanratchakool Aquatic Animal Health Research Institute Department of Fisheries Kasetsart University Campus Jatujak, Ladyao, Bangkok 10900 **THAILAND** Tel: 662-5794122 Fax: 662-5613993 E-mail: pornlerc@fisheries.go.th
	Mr. Daniel F. Fegan National Center for Genetic Engineering and Biotechnology (BIOTEC) Shrimp Biotechnology Programme 18th Fl. Gypsum Buidling Sri Ayuthya Road, Bangkok **THAILAND** Tel: 662-261-7225 Fax:662-261-7225 E-mail: dfegan@usa.net
	Dr. Chalor Limsuan Chalor Limsuwan Faculty of Fisheries, Kasetsart Unviersity Jatujak, Bangkok 10900 **THAILAND** Tel: 66-2-940-5695

Annex C.AII. List of Regional Resource Experts for Crustacean Diseases in Asia-Pacific

Shrimp Viruses	**Dr. Gary Nash** Center for Excellence for Shrimp Molecular Biology and Biotechnology Chalerm Prakiat Building Faculty of Science, Mahidol University Rama 6 Road Bangkok 10400 **THAILAND** Tel: 66-2-201-5870 to 5872 Fax: 66-2-201-5873 E-mail: gnash@asiaaccess.net.th
	Dr Nguyen Thanh Phuong Aquaculture and Fisheries Sciences Institute (AFSI) College of Agriculture Cantho University, Cantho **VIETNAM** Tel.: 84-71-830-931/830246 Fax: 84-71-830-247. E-mail: ntphuong@ctu.edu.vn
	Dr Peter Walker Associate Professor and Principal Research Scientist CSIRO Livestock Industries PMB 3 Indooroopilly Q 4068 **AUSTRALIA** Tel: 61 7 3214 3758 Fax: 61 7 3214 2718 E-mail : peter.walker@tag.csiro.au
Bacterial Diseases	**Mrs P.K.M. Wijegoonawardena** National Aquatic Resources Research and Development Agency Colombo 15, **SRI LANKA** Tel: 941-522005 Fax: 941-522932 E-mail: priyanjaliew@hotmail.com
	Prof. Tim Flegel Centex Shrimp, Chalerm Prakiat Building Faculty of Science, Mahidol University Rama 6 Road, Bangkok 10400 **THAILAND** Personal Tel: (66-2) 201-5876 Office Tel: (66-2) 201-5870 or 201-5871 or 201-5872 Fax: (66-2) 201-5873 Mobile Phone: (66-1) 403-5833 E-mail: sctwf@mahidol.ac.th
	Mrs. Celia Lavilla-Torres Fish Health Section Aquaculture Department Southeast Asian Fisheries Development Center Tigbauan, Iloilo 5021 **PHILIPPINES** Tel: 63 33 335 1009 Fax: 63 33 335 1008 E-mail: celiap@aqd.seafdec.org.ph

ANNEX C.AIII. LIST OF USEFUL DIAGNOSTIC MANUALS/GUIDES TO CRUSTACEAN DISEASES IN ASIA-PACIFIC

Asian Fish Health Bibliography III Japan by Wakabayashi H (editor). Fish Health Special Publication No. 3. Japanese Society of Fish Pathology, Japan and Fish Health Section of Asian Fisheries Society, Manila, Philippines
Information: Japanese Society of Fish Pathology

Manual for Fish Diseases Diagnosis: Marine Fish and Crustacean Diseases in Indonesia (1998) by Zafran, Des Roza, Isti Koesharyani, Fris Johnny and Kei Yuasa
Information: Gondol Research Station for Coastal Fisheries
P.O. Box 140 Singaraja, Bali, Indonesia
Tel: (62) 362 92278
Fax: (62) 362 92272

Health Management in Shrimp Ponds. Third Edition (1998) by P. Chanratchakool, J.F.Turnbull, S.J.Funge-Smith,I.H. MacRae and C. Limsuan.
Information: Aquatic Animal Health Research Institute
Department of Fisheries
Kasetsart University Campus
Jatujak, Ladyao, Bangkok 10900
THAILAND
Tel: (66.2) 579.41.22
Fax: (66.2) 561.39.93
E-mail: aahri@fisheries.go.th

Fish Health for Fishfarmers (1999) by Tina Thorne
Information: Fisheries Western Australia
3rd Floor, SGIO Atrium
186 St. Georges Terrace, Perth WA 6000
Tel: (08) 9482 7333 Fax: (08) 9482 7389
Web: http://www.gov.au.westfish

Australian Aquatic Animal Disease – Identification Field Guide (1999) by Alistair Herfort and Grant Rawlin
Information: AFFA Shopfront – Agriculture, Fisheries and Forestry – Australia
GPO Box 858, Canberra, ACT 2601
Tel: (02) 6272 5550 or free call: 1800 020 157
Fax: (02) 6272 5771
E-mail: shopfront@affa.gov.au

Diseases in Penaeid Shrimps in the Philippines. Second Edition (2000). By CR Lavilla-Pitogo, G.D. Lio-Po, E.R. Cruz-Lacierda, E.V. Alapide-Tendencia and L.D. de la Pena
Information: Fish Health Section
SEAFDEC Aquaculture Department
Tigbauan, Iloilo 5021, Philippines
Fax: 63-33 335 1008
E-mail: aqdchief@aqd.seafdec.org.ph
devcom@aqd.seafdec.org.ph

Manual for Fish Disease Diagnosis - II: Marine Fish and Crustacean Diseases in Indonesia (2001) by Isti Koesharyani, Des Roza, Ketut Mahardika, Fris Johnny, Zafran and Kei Yuasa, edited by K. Sugama, K. Hatai, and T Nakai
Information: Gondol Research Station for Coastal Fisheries
P.O. Box 140 Singaraja, Bali, Indonesia
Tel: (62) 362 92278
Fax: (62) 362 92272

Annex C.AIII.List of Useful Diagnostic Manuals/ Guides to Crustacean Diseases in Asia-Pacific

Reference PCR Protocol for Detection of White Spot Syndrome Virus (WSSV) in Shrimp. Shrimp Biotechnology Service Laboratory. Vol. 1, No. 1, March 2001

Information: Shrimp Biotechnology Service Laboratory
73/1 Rama 6 Rd., Rajdhewee, Bangkok 10400
Tel. (662) 644-8150
Fax: (662) 644-8107

LIST OF NATIONAL COORDINATORS*

Country	Name and Address
Australia	**Dr. Eva -Maria Bernoth** Manager, Aquatic Animal Health Unit, Office of the Chief Veterinary Officer Department of Agriculture, Fisheries and Forestry GPO Box 858, Canberra ACT 2601, Australia Fax: 61-2-6272 3150; Tel: 61-2-6272 4328 Email: Eva-Maria.Bernoth@affa.gov.au
	Dr. Alistair Herfort (Focal point for disease reporting) Aquatic Animal Health Unit , Office of the Chief Veterinary Officer Department of Agriculture, Fisheries and Forestry GPO Box 858, Canberra ACT 2601, Australia Fax: +61 2 6272 3150; tel: +61 2 6272 4009 E-mail: Alistair.Herfort@affa.gov.au
Bangladesh	**Dr. M. A. Mazid** Director General, Bangladesh Fisheries Research Institute (BFRI) Mymensingh 2201, Bangladesh Fax: 880-2-55259, Tel: 880-2-54874 E-mail: frifs@bdmail.net
Cambodia	**Mr. Srun Lim Song** Head, Laboratory Section, Department of Fisheries 186 Norodom Blvd.,P.O. Box 835, Phnom Penh, Cambodia Fax: (855) 23 210 565; Tel: (855) 23 210 565 E-mail: smallfish@bigpond.com.kh
China P.R.	**Mr. Wei Qi** Extension Officer, Disease Prevention and Control Division National Fisheries Technology Extension Centre, No. 18 Ministry of Agriculture Mai Zi dian Street, Chaoyang District, Beijing 100026, China Fax: 0086-1—65074250; Tel: 0086-10-65074250 E-mail: weiqi_moa@hotmail.com
	Prof. Yang Ningsheng (*Focal point for AAPQIS*) Director, Information Center, China Academy of Fisheries Science 150 Qingta Cun, South Yongding Road, Beijing 100039, China Fax: 86-010-68676685; Tel: 86-010-68673942 E-mail: ningsheng.yang@mh.bj.col.com.cn
DPR Korea	**Mr. Chong Yong Ho** Director of Fish Farming Technical Department Bureau of Freshwater Culture Sochangdong Central District, P.O.Box. 95 , Pyongyong, DPR Korea Fax- 850-2-814416; Tel- 3816001, 3816121

* The matrix provides a list of National Coordinators nominated by Governments and focal points for the *Asia-Pacific Quarterly Aquatic Animal Disease Reports*.

List of National Coordinators

Hong Kong China	**Dr. Roger S.M. Chong** National Coordinator and Fish Health Officer Agriculture, Fisheries and Conservation Department Castle Peak Veterinary Laboratory San Fuk Road, Tuen Mun New Territories, Hong Kong Fax: +852 2461 8412 Tel: + 852 2461 6412 E-mail: vfhoafd@netvigator.com
India	**Dr. AG Ponniah** Director National Bureau of Fish Genetic Resources Canal Ring Road, P.O. Dilkusha Lucknow-226 002, U.O., India Fax: (911-522) 442403; Tel: (91-522) 442403/442441 E-mail: nbfgr@1w1.vsnl.net.in; nbfgr@400.nicgw.nic.in
	Shri M.K.R. Nair Fisheries Development Commissioner
Indonesia	**Mr. Bambang Edy Priyono** National Coordinator (from September 2000) Head, Division of Fish Health Management Directorate General of Fisheries Jl. Harsono RM No. 3 Ragunan Pasar Minggu Tromol Pos No.: 1794/JKS Jakarta – 12550 Indonesia Tel: 7804116-119 Fax: 7803196 – 7812866 E-mail: dfrmdgf@indosat.net.id
Iran	**Dr. Reza Pourgholam** National Coordinator (from November 2000) Veterinary Organization Ministry of Jihad – E – Sazandegi Vali-ASR Ave S.J.Asad Abadi St PO Box 14155 – 6349 Tehran, Iran Tel: 8857007-8857193 Fax: 8857252
Japan	**Dr. Shunichi Shinkawa** Fisheries Promotion Division, Fishery Agency 1-2-1, Kasumigaseki Chiyoda-ku, Tokyo 100-8907, Japan Fax: 813-3591-1084; Tel: 813-350-28111(7365) E-mail: shunichi_shinkawa@nm.maff.go.jp
Lao PDR	**Mr. Bounma Luang Amath** Fisheries and Livestock Department Ministry of Agriculture, Forestry and Fisheries P.O. Box 811, Vientianne, Lao PDR TeleFax: (856-21) 415674; Tel: (856-21) 416932

[1] The experts included in this list have previously been consulted and agreed to provide valuable information and health advise concerning their particular expertise.

List of National Coordinators

Malaysia	**Mr. Ambigadevi Palanisamy** (from September 2001) National Coordinator Fisheries Research Institute Department of Fisheries Penang, Malaysia E-mail: ambigadevip@yahoo.com
	Dr. Ong Bee Lee (focal point for disease reporting) Head, Regional Veterinary Laboratory Services Department of Veterinary Services 8th & 9th Floor, Wisma Chase Perdana Off Jln Semantan 50630, Kuala Lumpur, Malaysia Fax: (60-3) 254 0092/253 5804; Tel: (60-3) 254 0077 ext.173 E-mail: ong@jph.gov.my
Myanmar	**Ms. Daw May Thanda Wint** Assistant Staff Officer, Aquatic Animal Health Section Department of Fisheries Sinmin Road, Alone Township , Yangon, Myanmar Fax: (95-01) 228-253; Tel: (95-01) 283-304/705-547
Nepal	**Mr. M. B. Pantha** Chief, District Agri Devt. Officer Dist Agric. Devt Office Janakpur, Dhanusha Nepal Fax: (977-1) 486895 E-mail: image@bhawani.wlink.com.np
Pakistan	**Rana Muhammad Iqbal** Assistant Fisheries Development Commissioner II Ministry of Food, Agriculture and Cooperatives R#310, B-Block, Islamabad, Government of Pakistan, Islamabad, Pakistan Fax: 92-051-9201246; Tel: 92-051-9208267
	Dr. Rukshana Anjum Assistant Fisheries Development Commissioner Ministry of Food, Agriculture and Livestock Government of Pakistan Fax No. 051 9221246
Philippines	**Dr. Joselito R. Somga** Aquaculturist II, Fish Health Section, BFAR 860 Arcadia Building, Quezon Avenue, Quezon City 1003 Fax: (632)3725055/4109987; Tel:(632) 3723878 loc206 or 4109988 to 89 E-mail: sssomga@edsamail.co.ph
Republic of Korea	**Dr. Mi-Seon Park** Director of Pathology Division National Fisheries Research and Development Institute 408-1 Sirang, Kijang Pusan 619-900 Korea RO Tel: 82-51-720-2470; Fax: 82-51-720-2498 E-mail: parkms@haema.nfrda.re.kr

List of National Coordinators

Singapore	**Mr. Chao Tien Mee** SAVAO (Senior Agri-Food and Veterinary Authority Officer) OIC, Marine Aquaculture Centre (MAC) Agri-Food & Veterinary Authority of Singapore (AVA) 300 Nicoll Drive, Changi Point, Singapore 498989 Tel: (65) 5428455; Fax No.: (65) 5427696 E-mail: CHAO_Tien_Mee@ava.gov.sg
	Dr. Chang Siow Foong (focal person for disease reporting) Agri-Food and Veterinary Authority of Singapore Central Veterinary Laboratory 60 Sengkang East Way Singapore 548596 Tel: (65) 3863572; Fax No. (65) 3862181 E-mail: CHANG_Siow_Foong@AVA.gov.sg
Sri Lanka	**Mr. A. M. Jayasekera** Director-General National Aquaculture Development Authority of Sri Lanka Ministry of Fisheries and Aquatic Resources Development, 317 1/1 T.B. Jayah Mawatha, Colombo 10, Sri Lanka Tel: (94-1) 675316 to 8; Fax: (94-1) 675437 E-mail: aqua1@eureka.lk
	Dr. Geetha Ramani Rajapaksa (focal point for disease reporting) Veterinary Surgeon Department of Animal Production and Health Veterinary Investigation Centre, Welisara, Ragama, Sri Lanka Tel: + 01-958213 E-mail: sser@sri.lanka.net
Thailand	**Dr. Somkiat Kanchanakhan** Fish Virologist, Aquatic Animal Health Research Institute (AAHRI) Department of Fisheries , Kasetsart University Campus Jatujak, Bangkok 10900, Thailand Fax: 662-561-3993; Tel: 662-579-4122, 6977 E-mail: somkiatk@fisheries.go.th
Vietnam	**Dr. Le Thanh Luu** Vice-Director Research Institute for Aquaculture No. 1 (RIA No. 1) Dinh Bang, Tien Son, Bac Ninh, Vietnam Fax: 84-4-827-1368; Tel: 84-4-827-3070 E-mail: ria1@hn.vnn.vn
	Ms Dang Thi Lua (Focal point for disease reporting) Researcher, Research Institute for Aquaculture No.1 (RIA No.1) Dinh Bang , Tien Son, Bac Ninh, Vietnam Fax: 84-4-827-1368; Tel : 84-4-827 - 3070 E-mail: ria1@hn.vnn.vn; danglua@hotmail.com

MEMBERS OF THE REGIONAL WORKING GROUP

(RWG, 1998 TO 2001)

Dr. Eva-Maria Bernoth
Manager, Aquatic Animal Health Unit
Office of the Chief Veterinary Officer
Department of Agriculture, Fisheries and Forestry – Australia
GPO Box 858, Canberra Act 2601
AUSTRALIA
Tel: 61-2-6272-4328
Fax: 61-2-6272-3150
E-mail: Eva-Maria.Bernoth@affa.gov.au

Mr. Daniel Fegan
Apt. 1D Prestige Tower B
168/25 Sukhumvit Soi 23
Klongtoey, Bangkok 10110
THAILAND
Tel: (662) 261-7225/(661) 825-8714
Fax: (662) 261-7225
E-mail: dfegan@usa.net

Professor Jiang Yulin
Shenzhen Exit & Entry Inspection and Quarantine Bureau
40 Heping Road, Shenzhen 518010
PEOPLE'S REPUBLIC OF CHINA
Tel: 86-755-5592980
Fax: 86-755-5588630
E-mail: szapqbxi@public.szptt.net.cn

Dr. Indrani Karunasagar
UNESCO MIRCEN for Marine Biotechnology
Department of Fishery Microbiology
University of Agricultural Sciences
College of Fisheries
Mangalore – 575 002
Karnataka
INDIA
Tel: 91-824 436384
Fax: 91-824 436384/91-824 438366
E-mail: mircen@giasbg01.vsnl.net.in

Ms. Celia Lavilla-Pitogo Torres
SEAFDEC Aquaculture Department
5021 Tigbauan
REPUBLIC OF THE PHILIPPINES
Tel: 63-33 336 2965
Fax: 63-33 335 1008 E-mail: celiap@seafdec.org.ph

Professor Mohammed Shariff
Faculty of Veterinary Medicine
Universiti Putra Malaysia
43400 Serdang, Selangor Darul Ehsan
MALAYSIA
Tel: 60-3-89431064
Fax: 60-3-89430626
E-mail: shariff@vet.upm.edu.my

Members of the Regional Working Group (RWG, 1998 to 2001)

Dr. Kamonporn Tonguthai
Department of Fisheries
Kasetsart University Campus
Ladyao, Jatujak, Bangkok 10900
THAILAND
Tel: (662) 940-6562
Fax: (662) 562-0571
E-mail: kamonpot@fisheries.go.th

Dr. Yugraj Singh Yadava
National Agriculture Technology Project
Ministry of Agriculture
Pusa, New Delhi 110012
INDIA
Tel: (91-11)-6254812 (residence)
(91-11) 5822380/5822381 (office)
E-mail: y.yugraj@mailcity.com

MEMBERS OF THE TECHNICAL SUPPORT SERVICES:

Dr. James Richard Arthur
FAO Consultant
RR1, Box 13, Savarie Rd.
Sparwood, B.C.
Canada V0B 2G0
Tel: 250-425-2287
Fax: 250 425-0045 (indicate for delivery to R. Arthur, Tel. 425-2287)
E-mail: rarthur@titanlink.com

Dr. Chris Baldock
Director, AusVet Animal Health Services
PO Box 3180
South Brisbane Qld 4101
AUSTRALIA
Tel: 61-7-3255 1712
Fax: 61-7-3511 6032
E-mail: ausvet@eis.net.au

Mr. Pedro Bueno
Co-ordinator
Network of Aquaculture Centres in
Asia-Pacific
Department of Fisheries
Kasetsart University Campus
Ladyao, Jatujak, Bangkok 10900
THAILAND
Tel: (662) 561-1728 to 9
Fax: (662) 561-1727
E-mail: pedro.bueno@enaca.org

Dr. Supranee Chinabut
Director, Aquatic Animal Health Research Institute
Department of Fisheries
Kasetsart University Campus,
Ladyao, Jatujak, Bangkok 10900
THAILAND
Tel: (662) 579-4122
Fax: (662) 561-3993
E-mail: supranee@fisheries.go.th

Professor Timothy Flegel
Department of Biotechnology
Faculty of Science
Mahidol University
Rama 6 Road, Bangkok 10400
THAILAND
Tel: (662) 245-5650
Fax: (662) 246-3026
E-mail: sctwf@mahidol.ac.th

Professor Tore Hastein
National Veterinary Institute
Ullevalsveien 68,
P.O. Box 8156 Dep. 0033
NORWAY
Tel: 47 22964710
Fax: 47 22463877
E-mail: Tore.Hastein@vetinst.no

Members of the Technical Support Services:

Dr. Barry Hill
OIE Fish Disease Commission
CEFAS Weymouth Laboratory
The Nothe, Weymouth, Dorset DT4 8UB
UNITED KINGDOM
Tel: 44-1305 206 626
Fax: 44-1305-206 627
E-mail: B.J.HILL@cefas.co.uk

Mr. Hassanai Kongkeo
Special Advisor Network of Aquaculture Centres in Asia-Pacific
Department of Fisheries
Kasetsart University Campus
Ladyao, Jatujak, Bangkok 10900
THAILAND
Tel: (662) 561-1728 to 9
Fax: (662) 561-1727
E-mail: hassanak@fisheries.go.th

Dr. Sharon E. McGladdery
Shellfish Health Pathologist
Department of Fisheries and Oceans – Canada
Gulf Fisheries Centre, P.O. Box 5030
Moncton, NB, CANADA E1C 9B6
Tel: 506 851-2018
Fax: 506 851-2079
E-mail: McGladderyS@mar.dfo-mpo.gc.ca

Dr. Kazuhiro Nakajima
Head, Pathogen Section
Fish Pathology Division
National Research Institute of Aquaculture
422-1 Nansei-cho, Watarai-gun
Mie 516-0193
JAPAN
Tel: 81-599 66-1830
Fax: 81-599 6 6-1962
E-mail: kazuhiro@nria.affrc.go.jp

Dr. Yoshihiro Ozawa
OIE Representation for Asia and the Pacific
OIE Tokyo, East 311, Shin Aoyama Bldg
1-1-1 Minami-aoyama, Minato-ku
Tokyo 107
JAPAN
Tel: 81-3-5411-0520
Fax: 81-3-5411-0526
E-mail: Oietokyo@tky.3web.ne.jp

Dr. Michael J. Phillips
Environment Specialist
Network of Aquaculture Centres in
Asia-Pacific
Department of Fisheries
Kasetsart University Campus
Ladyao, Jatujak, Bangkok 10900
THAILAND
Tel: (662) 561-1728 to 9
Fax: (662) 561-1727

Members of the Technical Support Services:

E-mail: Michael.Phillips@enaca.org

Dr. Melba B. Reantaso
Aquatic Animal Health Specialist
Network of Aquaculture Centres in
Asia-Pacific
Department of Fisheries, Kasetsart University Campus
Ladyao, Jatujak, Bangkok 10900
THAILAND
Tel: (662) 561-1728 to 9
Fax: (662) 561-1727
E-mail: Melba.Reantaso@enaca.org; melbar@fisheries.go.th

Dr. Rohana P. Subasinghe
Senior Fishery Resources Officer
(Aquaculture)
Inland Water Resources and
Aquaculture Service
Fisheries Department,
Food and Agriculture Organization of
the United Nations
Viale delle Terme di Caracalla
Rome 00100
ITALY
Tel: 39-06 570 56473
Fax: 39-06 570 53020
E-mail: Rohana.Subasinghe@fao.org

Mr. Zhou Xiao Wei
Program Officer (Training)
Network of Aquaculture Centres in
Asia-Pacific
Department of Fisheries
Kasetsart University Campus
Ladyao, Jatujak, Bangkok 10900
THAILAND
Tel: (662) 561-1728 to 9
Fax: (662) 561-1727
E-mail: zhoux@fisheries.go.th

LIST OF FIGURES

SECTION 2 – FINFISH DISEASES

SECTION F.1 GENERAL TECHNIQUES

Fig. F.1.1.2.1a. Red spot disease of grass carp **(MG Bondad-Reantaso)**
Fig. F.1.1.2.1b. Surface parasites, *Lerneae cyprinacea* infection of giant gouramy **(JR Arthur)**
Fig. F.1.1.2.1c. Ayu, *Plecoglossus altivelis*, infected with *Posthodiplostomum cuticola* (?) metacercariae appearing as black spots on skin **(K Ogawa)**
Fig. F.1.1.2.1d. Typical ulcerative, popeye, fin and tail rot caused by *Vibrio spp.* **(R Chong)**
Fig. F.1.1.2.2a. Example of gill erosion on Atlantic salmon, *Salmo salar*, due to intense infestation by the copepod parasite *Salmincola salmoneus* **(SE McGladdery)**
Fig. F.1.1.2.2b. Fish gills infected with monogenean parasites **(MG Bondad-Reantaso)**
Fig. F.1.3.1a. *Myxobolus artus* infection in the skeletal muscle of 0+ carp **(H Yokoyama)**
Fig. F.1.3.1b. *Ligula* sp. (Cestoda) larvae infection in the body cavity of Japanese yellow goby, *Acanthogobius flavimanus* **(K Ogawa)**
Fig. F.1.3.2a. Distended abdomen of goldfish **(H Yokoyama)**
Fig. F.1.3.2b. Japanese Yamame salmon *(Onchorynchus masou)* fingerlings showing swollen belly due to yeast infection **(MG Bondad-Reantaso)**

SECTION F.2 – EPIZOOTIC HAEMATOPOIETIC NECROSIS (EHN)

Fig. F.2.2. Mass mortality of single species of redfin perch. Note the small size of fish affected and swollen stomach of the individual to the center of the photograph. Note the characteristic haemorrhagic gills in the fish on the left in the inset **(AAHL)**

SECTION F.3 – INFECTIOUS HAEMATOPOIETIC NECROSIS (IHN)

Fig.F.3.2a. IHN infected fry showing yolk sac haemorrhages **(EAFP)**
Fig.F.3.2b. Clinical signs of IHN infected fish include darkening of skin, haemorrhages on the abdomen and in the eye around the pupil **(EAFP)**

SECTION F.4 *ONCORHYNCHUS MASOU* VIRUS (OMV)

Fig. F.4.4.1.1a. OMV-infected chum salmon showing white spots on the liver **(M Yoshimizu)**
Fig. F.4.4.1.1b. OMV-induced tumour developing around the mouth of chum salmon fingerling **(M Yoshimizu)**
Fig. F.4.4.1.3. OMV particles isolated from masou salmon, size of nucleocapsid is 100 to 110 *nm* **(M Yoshimizu)**

SECTION F.5 INFECTIOUS PANCREATIC NECROSIS (IPN)

Fig.F.5.2a. IPN infected fish showing dark colouration of the lower third of the body and small swellings on the head **(EAFP)**
Fig.F.5.2b. Rainbow trout fry showing distended abdomen characteristic of IPN infection. Eyed-eggs of this species were imported from Japan into China in 1987 **(J Yulin)**
Fig.F.5.2c. Top: normal rainbow trout fry, below: diseased fry **(EAFP)**
Fig. F.5.4.1.3. CPE of IHNV **(J Yulin)**
Fig.F.5.4.1.4. IPN virus isolated from rainbow trout fry imported from Japan in 1987. Virus particles are 55 *nm* in diameter **(J Yulin)**

SECTION F.6 VIRAL ENCEPHALOPATHY AND RETINOPATHY (VER)

Fig.F.6.2. Fish mortalities caused by VER (J Yulin)
Figs. F.6.4.1.2a, b. Vacuolation in brain (Br) and retina (Re) of GNNV-infected grouper in Chinese Taipei (bar = 100 mm) **(S Chi Chi)**

List Of Figures

SECTION F.7 SPRING VIRAEMIA OF CARP (SVC)

Figs.F.7.4.1.1a, b,c,d. Non-specific clinical signs of SVC infected fish, which may include swollen abdomen, haemorrhages on the skin, abdominal fat tissue, swim bladder and other internal organs and in the muscle **(EAFP)**

SECTION F.8 VIRAL HAEMORRHAGIC SEPTICAEMIA (VHS)

Fig.F.8.4.1.1. Non-specific internal sign (petechial haemorrhage on muscle) of VHS infected fish **(EAFP)**

SECTION F.9 LYMPHOCYSTIS

Fig.F.9.2a. Wild snakehead infected with lymphocystis showing irregularly elevated masses of pebbled structure **(MG Bondad-Reantaso)**
Fig.F.9.4.1.1a. Flounder with severe lymphocystis **(J Yulin)**
Fig.F.9.4.1.1b. Lymphocystis lesions showing granular particle inclusions **(J Yulin)**
Fig.F.9.4.1.1c. Carp Pox Disease caused by Herpesvirus **(J Yulin)**
Fig.F.9.4.1.1d. Goldfish with fungal (mycotic) skin lesions **(J Yulin)**
Fig.F.9.4.2.1a. Giant (hypertrophied) lymphoma cells with reticulate or branching inclusion bodies around the nuclei **(J Yulin)**
Fig.F.9.4.2.1b. Impression smear of lymphocystis showing some giant cells, and hyaline capsules (membrane) **(J Yulin)**
Fig.F.9.4.2.2a. Electron micrograph showing numerous viral particles in cytoplasm **(J Yulin)**
Fig. F.9.4.2.2b. Enlarged viral particles showing typical morphology of iridovirus (100 mm = bar) **(J Yulin)**
Fig.F.9.4.2.2c. *Herpervirus* in Carp Pox showing enveloped and smaller virions compared to lymphocystis virus **(J Yulin)**

SECTION F.10 BACTERIAL KIDNEY DISEASE (BKD)

Fig.F.10.3.1.2a. Pinpoint colonies up to 2 mm in diameter of *Renibacteriium salmonimarum*, white-creamy, shiny, smooth, raised and entire; three weeks after incubation at 15°C on KDM-2 medium **(M Yoshimizu)**
Fig.F.10.3.1.2b. *Renibacterium salmoninarum* rods, isolated from masou salmon **(M Yoshimizu)**
Fig.F.10.4.1.1a. Kidney of masou salmon showing swelling with irregular grayish patches **(M Yoshimizu)**
Fig.F.10.4.1.1b. Enlargement of spleen is also observed from BKD infected fish **(EAFP)**

SECTION F.11 EPIZOOTIC ULCERATIVE SYNDROME (EUS)

Fig.F.11.1.2a. Ayu, *Plecoglatus altivelis*, infected with mycotic granulomatosis **(K Hatai)**
Fig.F.11.1.2b. EUS affected farmed silver perch *Bidyanus bidyanus* from Eastern Australia **(RB Callinan)**
Fig.F.11.2a. Catfish showing initial EUS red spots **(MG Bondad-Reantaso)**
Fig.F.11.2b. Snakehead in Philippines (1985) showing typical EUS lesions **(MG Bondad-Reantaso)**
Fig.F.11.4.1.1a. Wild mullet in Philippines (1989) with EUS **(MG Bondad-Reantaso)**
Fig.F.11.4.1.1b. Red spot disease of grass carp in Vietnam showing ulcerative lesions **(MG Bondad-Reantaso)**
Fig.F.11.4.1.2. Granuloma from squash preparation of muscle of EUS fish **(MG Bondad-Reantaso)**
Fig.F.11.4.2.1a. Typical severe mycotic granulomas from muscle section of EUS fish (H & E) **(MG Bondad-Reantaso)**
Fig.F.11.4.2.1b. Mycotic granulomas showing fungal hyphae (stained black) using Grocotts stain **(MG Bondad-Reantaso)**
Fig.F.11.4.2.2a. Typical characteristic of *Aphanomyces* sporangium **(K Hatai)**
Fig.F.11.4.2.2b. Growth of *Aphanomyces invadans* on GP agar **(MG Bondad-Reantaso)**

List Of Figures

SECTION 3 MOLLUSCAN DISEASES

SECTION M.1 GENERAL TECHNIQUES

Fig.M.1.1.1. Gaping hard shell clam, *Mercenaria mercenaria*, despite air exposure and mechnical tapping **(SE McGladdery)**
Fig.M.1.1.2a. Mollusc encrustment (arrows) of winged oyster, Pteria penguin, Guian Pearl Farm, Eastern Samar, Philippines (1996) **(MG Bondad-Reantaso)**
Fig.M.1.1.2b. *Pteria penguin* cultured at Guian Pearl Farm, Eastern Samar, Philippines with extensive shell damage due to clionid (boring) sponge (1992) **(D Ladra)**
Fig.M.1.1.2c,d. *Pteria penguin* shell with dense multi-taxa fouling, Guian Pearl Farm, Eastern Philippines (1996) **(MG Bondad-Reantaso)**
Fig.M.1.1.2e. *Polydora* sp. tunnels and shell damage at hinge of American oyster, *Crassostrea virginica*, plus barnacle encrusting of other shell surfaces **(SE McGladdery)**
Fig.M.1.1.2f. Winged oyster, *Pteria penguin*, shell with clionid sponge damage. Guian Pearl Farm, Eastern, Philippines (1996) **(MG Bondad-Reantaso)**
Fig.M.1.1.2g,h. *Pinctada maxima*, shell with clionid sponge damage due to excavation of tunnels exhalent-inhalent openings (holes) to the surface (arrows). Other holes (small arrows) are also present that may have been caused by polychaetes, gastropod molluscs or other fouling organisms. Guian Pearl Farm, Eastern Philippines (1996) **(MG Bondad-Reantaso)**
Fig.M.1.1.3a. Winged oyster, *Pteria penguin*, shell showing clionid sponge damage through to the inner shell surfaces, Guian Pearl Farm, Eastern Philippines (1996) **(MG Bondad-Reantaso)**
Fig.M.1.1.3a1. Abalone (*Haliotis roei*) from a batch killed by polydoriid worms **(B Jones)**
Fig.M.1.1.3b,c. b. Shells of *Pinctada maxima* showing a erosion of the nacreous inner surfaces (arrows), probably related to chronic mantle retraction; c. Inner surface of shell showing complete penetration by boring sponges (thin arrows) **(D Ladra)**
Fig.M.1.1.3d,e,f. *Pinctada maxima* (d), *Pteria penguin* (e) and edible oyster (*Crassostrea* sp.) (f) shells showing *Polydora*-related tunnel damage that has led to the formation of mud-filled blisters **(MG Bondad-Reantaso)**
Fig.M.1.1.3g. Inner shell of winged pearl oyster showing: tunnels at edge of the shell (straight thick arrow); light sponge tunnel excavation (transparent arrow); and blisters (small thick arrow) at the adductor muscle attachment site. Guian Pearl Farm, Eastern Philippines (1996)
(MG Bondad-Reantaso)
Fig.M.1.1.3.h. Extensive shell penetration by polychaetes and sponges causing weakening and retraction of soft-tissues away from the shell margin of an American oyster *Crassostrea virginica*
(SE McGladdery)
Fig.M.1.1.4a. Normal oyster (*Crassostrea virginica*) tissues **(SE McGladdery)**
Fig.M.1.1.4b. Watery oyster (*Crassostrea virginica*) tissues – compare with M.1.1.4a
(SE McGladdery)
Fig.M.1.1.4c. Abscess lesions (creamy-yellow spots) in the mantle tissue of a Pacific oyster (*Crassostrea gigas*) **(SE McGladdery)**
Fig.M.1.1.4d. Gross surface lesions in Pacific oyster (*Crassostrea gigas*) due to *Marteiliodes chungmuensis* **(MS Park and DL Choi)**
Fig.M.1.1.4e. Water blister (oedema/edema) in the soft-tissues of the mantle margin of an American oyster (*Crassostrea virginica*) **(SE McGladdery)**
Fig.M.1.1.4f. Calcareous deposits ("pearls") in the mantle tissues of mussels in response to irritants such as mud or digenean flatworm cysts **(SE McGladdery)**
Fig.M.1.1.4g. Polydoriid tunnels underlying the nacre at the inner edge of an American oyster (*Crassostrea virginica*) shell, plus another free-living polychaete, *Nereis diversicolor* on the inner shell surface **(SE McGladdery and M Stephenson)**

SECTION M.2 BONAMIOSIS

Fig.M.2.2a. Haemocyte infiltration and diapedesis across intestinal wall of a European oyster (*Ostrea edulis*) infected by *Bonamia ostreae* **(SE McGladdery)**
Fig.M.2.2b. Oil immersion of *Bonamia ostreae* inside European oyster (*Ostrea edulis*) haemocytes (arrows). Scale bar 20µm **(SE McGladdery)**
Fig.M.2.2c. Systemic blood cell infiltration in Australian flat oyster (*Ostrea angasi*) infected by

List Of Figures

Bonamia sp. Note vacuolised appearance of base of intestinal loop and duct walls (H&E) **(PM Hine)**
Fig.M.2.2d. Oil immersion of *Bonamia* sp. infecting blood cells and lying free (arrows) in the haemolymph of an infected Australian flat oyster, *Ostrea angasi*. Scale bar 20µm (H&E) **(PM Hine)**
Fig.M.2.2e. Focal infiltration of haemocytes around gut wall (star) of *Tiostrea lutaria* (New Zealand flat oyster) typical of infection by *Bonamia* sp. (H&E) **(PM Hine)**
Fig.M.2.2f. Oil immersion of haemocytes packed with *Bonamia* sp. (arrows) in an infected *Tiostrea lutaria* (H&E) **(PM Hine)**

SECTION M.3. MARTEILIOSIS

Fig.M.3.2a. Digestive duct of a European oyster, *Ostrea edulis*, showing infection of distal portion of the epithelial cells by plasmodia (arrows) of *Marteilia refringens*. Scale bar 15µm (H&E) **(SE McGladdery)**
Fig.M.3.2b. Digestive tubule of a European oyster, *Ostrea edulis*, showing refringent spore stage of *Marteilia refringens* (star). Scale bar 50µm (H&E) **(SE McGladdery)**
Fig.M.3.4.1.1a. Tissue imprint from *Saccostrea commercialis* (Sydney rock oyster) heavily infected by *Marteilia sydneyi* (arrows) (QX disease). Scale bar 250µm (H&E) **(RD Adlard)**
Fig.M.3.4.1.1b. Oil immersion of tissue squash preparation of spore stages of *Marteilia sydneyi* from Sydney rock oyster (*Saccostrea commercialis*) with magnified inset showing two spores within the sporangium. Scale bar 50µm (H&E) **(RD Adlard)**

SECTION M.4 MIKROCYTOSIS

Fig.M.4.2a. Gross abscess lesions (arrows) in the mantle tissues of a Pacific oyster (*Crassostrea virginica*) severely infected by *Mikrocytos mackini* (Denman Island Disease) **(SM Bower)**
Fig.M.4.3.2.1a. Histological section through mantle tissue abscess corresponding to the gross lesions pictured in Fig.M.4.2a, in a Pacific oyster (*Crassostrea gigas*) infected by *Mikrocytos mackini* (H&E) **(SM Bower)**
Fig.M.4.3.2.1b. Oil immersion of *Mikrocytos mackini* (arrows) in the connective tissue surrounding the abscess lesion pictured in Fig.M.4.3.2.1a. Scale bar 20µm (H&E) **(SM Bower)**

SECTION M.5. PERKINSOSIS

Fig.M.5.1.2a. *Arca* clam showing a *Perkinsus*-like parasite within the connective tissue. Magnified insert shows details of an advanced 'schizont' like stage with trophozoites showing vacuole-like inclusions. Scale bar 100µm. (H&E) **(PM Hine)**
Fig.M.5.1.2b. *Pinctada albicans* pearl oyster showing a *Perkinsus*-like parasite. Magnified insert shows details of a 'schizont'-like stage containing 'trophozoites' with vacuole-like inclusions. Scale bar 250µm (H&E) **(PM Hine)**
Fig.M.5.3.2.1a. Trophozoite ('signet-ring') stages of *Perkinsus marinus* (arrows), the cause of 'Dermo' disease in American oyster (*Crassostrea virginica*) connective tissue. Scale bar 20µm (H&E) **(SM Bower)**
Fig.M.5.3.2.1b. Schizont ('rosette') stages of *Perkinsus marinus* (arrows), the cause of 'Dermo' disease in American oyster (*Crassostrea virginica*) digestive gland connective tissue. Scale bar 30µm (H&E) **(SE McGladdery)**
Fig.5.3.2.2. Enlarged hypnospores of *Perkinsus marinus* stained blue-black with Lugol's iodine following incubation in fluid thioglycollate medium. Scale bar 200µm **(SE McGladdery)**

SECTION M.6 HAPLOSPORIDIOSIS

Fig.M.6.1.3a. Massive connective tissue and digestive tubule infection by an unidentified *Haplosporidium*-like parasite in the gold-lipped pearl oyster *Pinctada maxima* from north Western Australia. Scale bar 0.5 mm (H&E) **(PM Hine)**
Fig.M.6.1.3b. Oil immersion magnification of the operculated spore stage of the *Haplosporidium*-like parasite in the gold-lipped pearl oyster *Pinctada maxima* from north Western Australia. Scale bar 10 µm. (H&E) **(PM Hine)**

List Of Figures

Fig.M.6.1.3c. Haemocyte infiltration activity in the connective tissue of a Sydney rock oyster (*Saccostrea cucullata*) containing spores of a *Haplosporidium*-like parasite (arrow). Scale bar 0.5 mm. (H&E) **(PM Hine)**
Fig.M.6.1.3d. Oil immersion magnification of *Haplosporidium*-like spores (arrow) associated with heavy haemocyte infiltration in a Sydney rock oyster (*Saccostrea cucullata*). Scale bar 10µm. (H&E) **(PM Hine)**
Fig.M.6.3.1.2a. Plasmodia (black arrows) and spores (white arrows) of *Haplosporidium costale*, the cause of SSO disease, throughout the connective tissue of an American oyster (*Crassostrea virginica*). Scale bar 50µm **(SE McGladdery)**
Fig.M.6.3.1.2b. Plasmodia (black arrows) and spores (white arrows) of *Haplosporidium nelsoni*, the cause of MSX disease, throughout the connective tissue and digestive tubules of an American oyster (*Crassostrea virginica*). Scale bar 100µm **(SE McGladdery)**
Fig.M.6.4.2.2a. Oil immersion magnification of SSO spores in the connective tissue of an American oyster *Crassostrea virginica*. Scale bar 15µm **(SE McGladdery)**
Fig.M.6.4.2.2b. Oil immersion magnification of MSX spores in the digestive tubule epithelium of an American oyster *Crassostrea virginica*. Scale bar 25µm. (H&E) **(SE McGladdery)**

SECTION M.7 MARTEILIODOSIS

Fig.M.7.2a,b. a. Gross deformation of mantle tissues of Pacific oyster (*Crassostrea gigas*) from Korea, due to infection by the protistan parasite *Marteiloides chungmuensis* causing retention of the infected ova within the ovary and gonoducts; b. (insert) normal mantle tissues of a Pacific oyster **(MS Park and DL Choi)**
Fig.M.7.4.2.1. Histological section through the ovary of a Pacific oyster (*Crassostrea gigas*) with normal ova (white arrows) and ova severely infected by the protistan parasite *Marteiliodes chungmuensis* (black arrows). Scale bar 100µm **(MS Park)**

SECTION 4 CRUSTACEAN DISEASES

SECTION 4.1 GENERAL TECHNIQUES

Fig.C.1.1.1.3a. Behaviour observation of shrimp PL in a bowl **(P Chanratchakool)**
Fig.C.1.1.1.3b. Light coloured shrimp with full guts from a pond with healthy phytoplankton **(P Chanratchakool)**
Fig.C.1.1.2.1a. Black discoloration of damaged appendages **(P Chanratchakool)**
Fig.C.1.1.2.1b. Swollen tail due to localized bacterial infection **(P Chanratchakool)**
Fig.C.1.1.2.2a,b. Shrimp with persistent soft shell **(P Chanratchakool/MG Bondad-Reantaso)**
Fig.C.1.1.2.3a. Abnormal blue and red discoloration **(P Chanratchakool)**
Fig.C.1.1.2.3b. Red discoloration of swollen appendage **(P Chanratchakool)**
Fig.C.1.1.3a. Severe fouling on the gills **(P Chanratchakool)**
Fig.C.1.1.3b. Brown discolouration of the gills **(P Chanratchakool)**
Fig.C.1.1.3c. Shrimp on left side with small hepatopancreas **(P Chanratchakool)**
Fig.C.1.2a, b, c. Examples of different kinds of plankton blooms (a- yellow/green coloured bloom; b- brown coloured bloom; c- blue-green coloured bloom **(P Chanratchakool)**
Fig.C.1.2d. Dead phytoplankton **(P Chanratchakool)**
Fig. C.1.3.6. Points of injection of fixative **(V Alday de Graindorge and TW Flegel)**

SECTION C.2 YELLOWHEAD DISEASE (YHD)

Fig.C.2.2. Gross sign of yellow head disease (YHD) are displayed by the three *Penaeus monodon* on the left **(TW Flegel)**
Fig.C.2.3.1.4a,b. Histological section of the lymphoid organ of a juvenile *P. monodon* with severe acute YHD at low and high magnification. A generalized, diffuse necrosis of LO cells is shown. Affected cells display pyknotic and karyorrhectic nuclei. Single or multiple perinuclear inclusion bodies, that range from pale to darkly basophilic, are apparent in some affected cells (arrows). This marked necrosis in acute YHD distinguishes YHD from infections due to Taura syndrome virus,which produces similar cytopathology in other target tissues but not in the LO. Mayer-Bennett H&E. 525x and 1700x magnifications, respectively **(DV Lightner)**

List Of Figures

Fig.C.2.3.1.4c. Histological section of the gills from a juvenile *P. monodon* with YHD. A generalized diffuse necrosis of cells in the gill lamellae is shown, and affected cells display pyknotic and karyorrhectic nuclei (arrows). A few large conspicuous, generally spherical cells with basophilic cytoplasm are present in the section.These cells may be immature hemocytes, released prematurely in response to a YHV-induced hemocytopenia. Mayer-Bennett H&E. 1000x magnification **(DV Lightner)**

SECTION C.3 INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS (IHHN)

Fig.C.3.2a. A small juvenile *Penaeus stylirostris* showing gross signs of acute IHHN disease. Visible through the cuticle, especially on the abdomen, are multifocal white to buff colored lesions in the cuticular epithelium or subcutis (arrows). While such lesions are common in *P. stylirostris* with acute terminal IHHN disease, they are not pathognomonic for IHHN disease **(DV Lightner)**
Fig.C.3.2b. Dorsal view of juvenile *P. vannamei* (preserved in Davidson's AFA) showing gross signs of IHHNV-caused RDS. Cuticular abnormalities of the sixth abdominal segment and tail fan are illustrated **(DV Lightner)**
Fig.C.3.2c. Lateral view of juvenile *P. vannamei* (preserved in Davidson's AFA) showing gross signs of IHHNV-caused RDS. Cuticular abnormalities of the sixth abdominal segment and tail fan are illustrated **(DV Lightner)**
Fig.C.3.4.1.2a. A low magnification photomicrograph (LM) of an H&E stained section of a juvenile *P. stylirostris* with severe acute IHHN disease. This section is through the cuticular epithelium and subcuticular connective tissues just dorsal and posterior to the heart. Numerous necrotic cells with pyknotic nuclei or with pathognomonic eosinophilic intranuclear inclusion bodies (Cowdry type A) are present (arrows). Mayer-Bennett H&E. 830x magnification **(DV Lightner)**
Fig.C.3.4.1.2b. A high magnification of gills showing eosinophilic intranuclear inclusions (Cowdry type A inclusions or CAIs) that are pathognomonic for IHHNV infections. Mayer-Bennett H&E. 1800x magnification **(DV Lightner)**

SECTION C.4 WHITE SPOT DISEASE (WSD)

Fig.C.4.2a. A juvenile *P. monodon* with distinctive white spots of WSD (DV Lightner)
Fig.C.4.2b. Carapace from a juvenile *P. monodon* with WSD. Calcareous deposits on the underside of the shell account for the white spots **(DV Lightner/P. Saibaba)**
Fig.C.4.3.3.1.2a. Histological section from the stomach of a juvenile *P.chinensis* infected with WSD. Prominent intranuclear inclusion bodies are abundant in the cuticular epithelium and subcuticular connective tissue of the organ (arrows) **(DV Lightner)**
Fig.C.4.3.3.1.2b. Section of the gills from a juvenile *P. chinensis* with WSBV. Infected cells show developing and fully developed intranuclear inclusion bodies of WSBV (arrows). Mayer-Bennett H&E. 900x magnification **(DV Lightner)**

SECTION C.4a BACTERIAL WHITE SPOT SYNDROME (BWSS)

Fig. C.4a.2. *Penaeus monodon* dense white spots on the carapace induced by WSD (M. Shariff)
Fig. C.4a.4.2.2a, b. Bacterial white spots (BWS), which are less dense than virus-induced white spots. Note some BWS have a distinct whitish marginal ring and maybe with or without a pinpoint whitish dot in the center **(M. Shariff/ Wang *et al.* 2000 (DAO 41:9-18))**
Fig. C.4a.4.2.2c. Presence of large number of bacteria attached to exposed fibrillar laminae of the endocuticle **(M. Shariff/ Wang *et al.* 2000 (DAO 41:9-18))**

SECTION C.5 BACULOVIRAL MIDGUT GLAND NECROSIS (BMGN)

Fig.C.5.1.2a. Section of the hepatopancreas of *P. plebejus* displaying several hepatopancreas cells containing BMN-type intranuclear inclusion bodies. Mayer-Bennett H&E. 1700 x magnification **(DV Lightner)**
Fig.C.5.4.2.1a. High magnification of hepatopancreas from a PL of *P. monodon* with a severe infection by a BMN-type baculovirus. Most of the hepatopancreas cells display infected nuclei. Mayer-Bennett H&E. 1700x magnification **(DV Lightner)**

List Of Figures

Fig. C.5.4.2.1b, c. Sections of the hepatopancreas of a PL of *P. japonicus* with severe BMN. Hepatopancreas tubules are mostly destroyed and the remaining tubule epithelial cells contain markedly hypertrophied nuclei that contain a single eosinophilic to pale basophilic, irregularly shaped inclusion body that fills the nucleus. BMNV infected nuclei also display diminished nuclear chromatin, marginated chromatin and absence of occlusion bodies that characterize infections by the occluded baculoviruses. Mayer-Bennett H&E. Magnifications: (a) 1300x; (b) 1700x **(DV Lightner)**
Fig.C.5.4.2.1d. MBV occlusion bodies which appear as esosinophilic, generally multiple, spherical inclusion bodies in enormously hypertrophied nuclei (arrows). Mayer-Bennett H&E. 1700x magnification **(DV Lightner)**

SECTION C.6 GILL-ASSOCIATED VIRUS (GAV)

Fig. C.6.4.2.1. Transmission electron microscopy of GAV **(P Walker)**

SECTION C.8 TAURA SYNDROME (TS)

Fig. C.8.4.1.1a,b. a. Moribund, juvenile, pond-reared *Penaeus vannamei* from Ecuador in the peracute phase of Taura Syndrome (TS). Shrimp are lethargic, have soft shells and a distinct red tail fan; b. Higher magnification of tail fan showing reddish discoloration and rough edges of the cuticular epithelium in the uropods suggestive of focal necrosis at the epithelium of those sites (arrows) **(DV Lightner)**
Fig. C.8.4.1.1c,d,e. Juvenile, pond-reared *P. vannamei* (c – from Ecuador; d – from Texas; e – from Mexico) showing melanized foci mark sites of resolving cuticular epithelium necrosis due to TSV infection **(DV Lightner/F Jimenez)**
Fig. C.8.4.1.2a. Focal TSV lesions in the gills (arrow). Nuclear pykinosis and karyorrhexis, increased cytoplasmic eosinophilia, and an abundance of variably staining generally spherical cytoplasmic inclusions are distinguishing characteristics of the lesions. 900x magnification **(DV Lightner)**
Fig. C.8.4.1.2b. Histological section through stomach of juvenile *P. vannamei* showing prominent areas of necrosis in the cuticular epithelium (large arrow). Adjacent to focal lesions are normal appearing epithelial cells (small arrows). Mayer-Bennett H&E. 300x magnification **(DV Lightner)**
Fig. C.8.4.1.2c. Higher magnification of Fig. C.8.4.1.2b showing the cytoplasmic inclusions with pyknotic and karyorrhectic nuclei giving a 'peppered' appearance. Mayer-Bennett H&E. 900x magnification **(DV Lightner)**
Fig. C.8.4.1.2d. Mid-sagittal section of the lymphoid organ (LO) of an experimentally infected juvenile *P. vannamei*. Interspersed among normal appearing lymphoid organ (LO) cords or tissue, which is characterized by multiple layers of sheath cells around a central hemolymph vessel (small arrow), are accumulations of disorganized LO cells that form LO 'spheroids". Lymphoid organs spheres (LOS) lack a central vessel and consists of cells which show karyomegaly and large prominent cytoplasmic vacuoles and other cytoplasmic inclusions (large arrow). Mayer-Bennett H&E. 300x magnification **(DV Lightner)**

SECTION C.9 NUCLEAR POLYHEDROSIS BACULOVIROSES (NPB)

Fig. C.9.3.2.1a. Wet mount of feces from a *P. vannamei* infected with BP showing tetrahedral occlusion bodies (arrows) which are diagnostic for infection of shrimp's hepatopancreas or midgut epithelial cells. Phase contrast, no stain. 700x magnification (DV Lightner)
Fig. C.9.3.2.1b,c. Mid and high magnification views of tissue squash preparations of the hepatopancreas (HP) from PL of *P. monodon* with MBV infections. Most HP cells in both PLs usually display multiple, generally spherical, intranuclear occlusion bodies (arrow) that are diagnostic for MBV. 0.1% malachite green. 700x (b) and 1 700x (c) magnifications **(DV Lightner)**

List Of Figures

Fig. C.9.3.2.3a,b. a. Mid-magnification view of mid-sagittal sections of PL of *P. vannamei* with severe BP infections of the hepatopancreas showing multiple eosinophilic BP tetrahedral occlusion bodies within markedly hypertrophied hepatopnacreas (HP) cell nuclei (arrows). Mayer-Bennett H&E. 700x magnification; b. High magnification of an HP tubule showing several BP-infected cells that illustrate well the intranuclear, eosinophilic, tetrahedral occlusion bodies of BP (arrows). Mayer-Bennett H&E. 1800x magnification **(DV Lightner)**

SECTION C.10 NECROTISING HEPATOPANCREATITIS (NH)

Fig. C.10.4.1.1. Juvenile *P. vannamei* with NHP showing markedly atrophied hepatopancreas, reduced to about 50% of its normal volume **(DV Lightner)**
Fig. C.10.4.1.2. Wet- mount of the HP of infected shrimp with inflamed hemocyte, melanized HP tubules and absence of lipid droplets. No stain. 150x magnification **(DV Lightner)**
Fig. C.10.4.1.3a,b. Low and mid-magnification of photographs of the HP of a severely NHP infected juvenile *P. vannamei.* Severe hemocytic inflammation of the intratubular spaces (small arrow) in response to necrosis, cytolysis and sloughing of HP tubule epithelial cells (large arrow), are among the principal histopathological changes due to NHP. Mayer-Bennett H&E. 150x (a) and 300x (b) magnifications **(DV Lightner)**
Fig. C.10.4.1.3c. Low magnification view of the HP of a juvenile P. vannamei with severe, chronic NHP. The HP tubule epithelium is markedly atrophied, resulting in the formation of large edematous (fluid filled or "watery" areas in the HP. Mayer-Bennett H & E. 100x magnification **(DV Lightner)**
Fig. C.10.4.1.3d. The HP tubule epithelial cells show no cytoplasmic lipid droplets, but instead contain masses of the tiny, non-membrane bound, intracytoplasmic NHP bacteria (arrow). Mayer-Bennett H&E. 1700x magnification **(DV Lightner)**
Fig. C.10.4.2.1. Low magnification TEM of a hepatopancreatocyte from a juvenile *P. vannamei* with NHP. Profiles of intracellular rod-shaped forms (large arrow) and helical forms (small arrow) of the NHP bacterium are abundant in the cytoplasm. 10 000x magnification **(DV Lightner)**

SECTION C.11 CRAYFISH PLAGUE

Fig. C.11.3.2.1a. Fresh microscopic mount of a piece of infected exoskeleton showing fungal spores **(EAFP/DJ Alderman)**
Fig. C.11.4.1.1a,b. Clinical signs of infected crayfish showing whitened necrotic musculature in the tail, and often accompanied in chronic infections by melanisation (blackening) of affected exoskeleton **(EAFP/DJ Alderman)**

The Asia Diagnostic Guide to Aquatic Animal Diseases or '*Asia Diagnostic Guide*' is an up- datable diagnostic guide for the pathogens and diseases listed in the NACA/FAO/ OIE Quarterly Aquatic Animal Disease Reporting System. It was developed from a large amount of technical contribution from aquatic animal health scientists in the Asia-Pacific region who supported the regional programme. The *Asia Diagnostic Guide*, which could be effectively used for both farm and laboratory level diagnosis in the region, not only complements the *Manual of Procedures* for the implementation of the Asia Regional *Technical Guidelines* on health management for the responsible movement of live aquatic animals, but also assists in expanding national and regional aquatic animal health diagnostic capabilities that will assist countries in upgrading technical capacities to meet the requirements in the OIE International Aquatic Animal Code and the OIE Diagnostic Manual for Aquatic Animal Diseases.

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