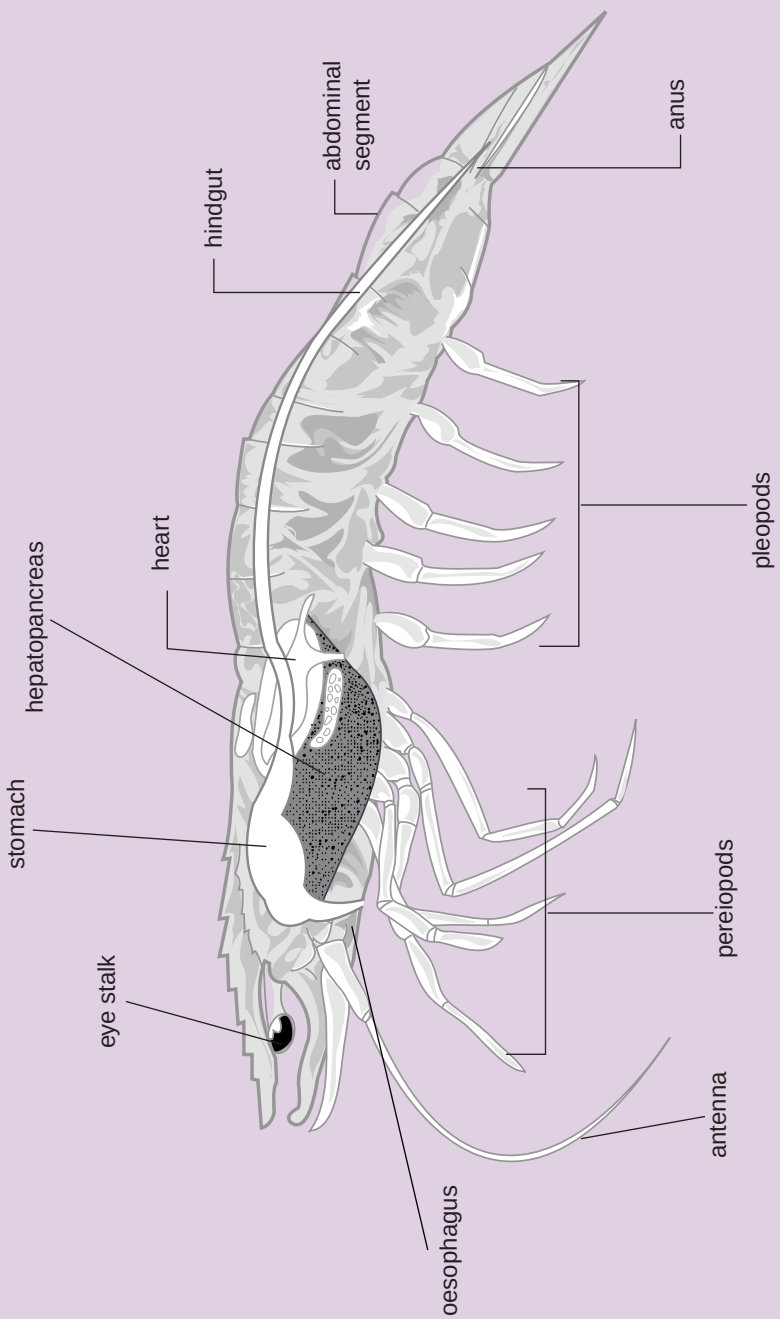
Internal and External Anatomy of a Penaeid Shrimp



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SECTION 4 - CRUSTACEAN DISEASES

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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.1.1.1.3 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

(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.

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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.

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Fig.C.1.1.3b. Brown discolouration of the gills.

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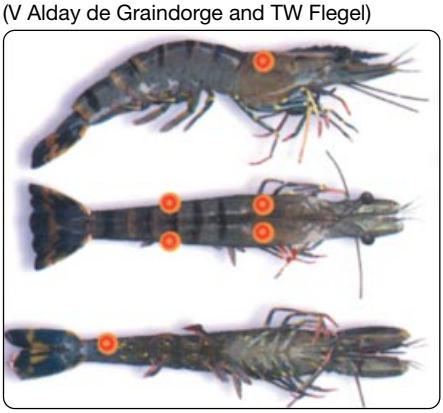
Fig.C.1.1.3c. Shrimp on left side with small hepatopancreas.

(V Alday de Graindorge and TW Flegel)



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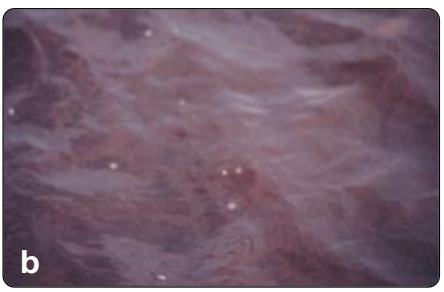

Fig.C.1.2a, b, c. Examples of different kinds of plankton blooms (a- yellow/green coloured bloom; b- brown coloured bloom; c- blue-green coloured bloom.

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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-

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 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 midabdominally. 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)

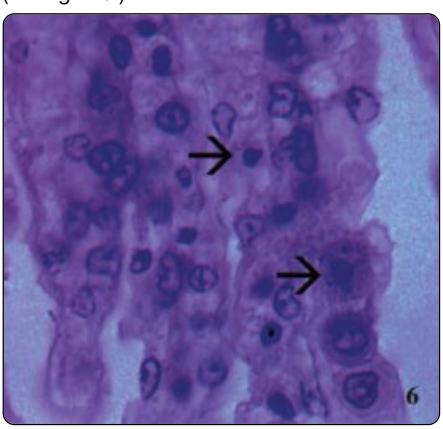
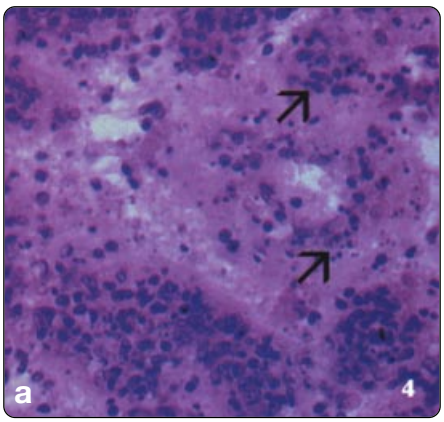


Fig.C.2.3.1.4c. Histological section of the gills from a juvenile *P. monodon* with YHD. A generalized diffuse necrosis of cells in the gill lamellae is shown, and affected cells display pyknotic and karyorrhectic nuclei (arrows). A few large conspicuous, generally spherical cells with basophilic cytoplasm 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)



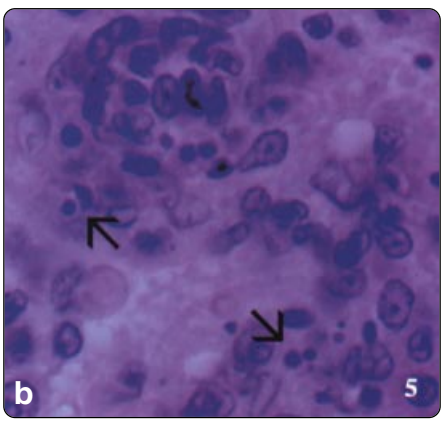


Fig.C.2.3.1.4a,b. Histological section of the lym phoid organ of a juvenile *P. monodon* with severe acute YHD at low and high magnification. A generalized, diffuse necrosis of LO cells is shown. Affected cells display pyknotic and karyorrhectic nuclei. Single or multiple perinuclear inclusion bodies, 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 subclinical 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)

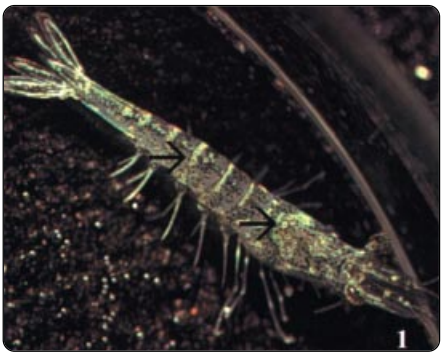


Fig.C.3.2a. A small juvenile *Penaeus stylirostris* showing gross signs of acute IHHN disease. Visible through the cuticle, especially on the abdomen, are multifocal white to buff colored lesions in the cuticular epithelium or subcutis (arrows). While such lesions are common in *P. stylirostris* with acute terminal IHHN disease, they are not pathognomonic for IHHN disease.

(DV Lightner)

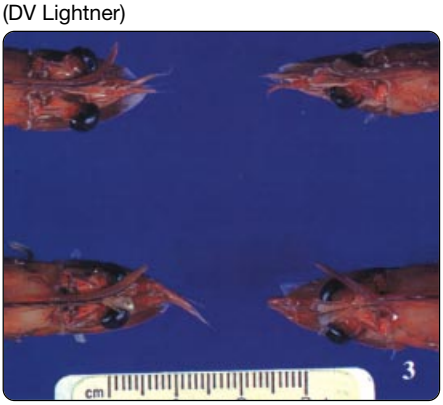


Fig.C.3.2b. Dorsal view of juvenile *P. vannamei* (preserved in Davidson's AFA) showing gross signs of IHHNV-caused RDS. Cuticular abnormalities of the sixth abdominal segment and tail fan are illustrated.

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)

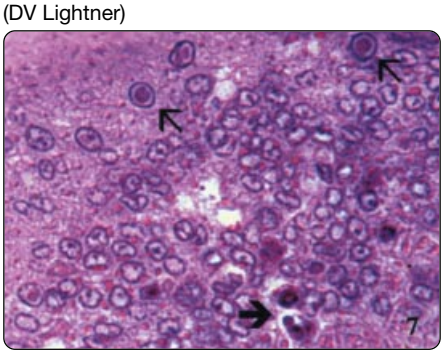
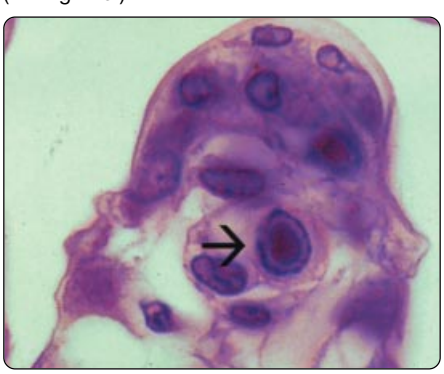


Fig.C.3.4.1.2b. A low magnification photomicrograph (LM) of an H&E stained section of a juvenile *P. stylirostris* with severe acute IHHN disease. This section is through the cuticular epithelium and subcuticular connective tissues just dorsal and posterior to the heart. Numerous necrotic cells with 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.

[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)

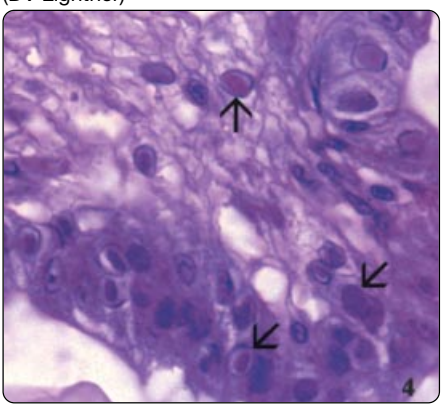

Fig.C.4.3.3.1.2a. Histological section from the stomach of a juvenile *P.chinensis* infected with WSD. Prominent intranuclear inclusion bodies are abundant in the cuticular epithelium and subcuticular connective tissue of the organ (arrows).

(DV Lightner)

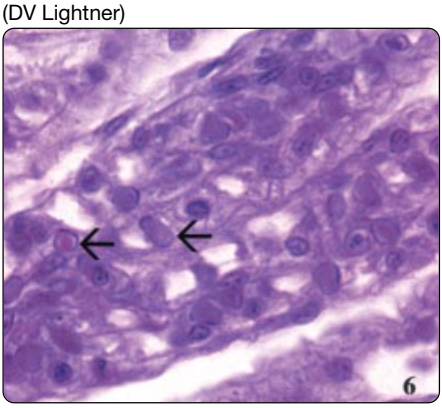

Fig.C.4.3.3.1.2b. Section of the gills from a juvenile *P. chinensis* with WSBV. Infected cells show developing and fully developed intranuclear inclusion bodies of WSBV (arrows). Mayer-Bennett H&E. 900x magnification.

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))

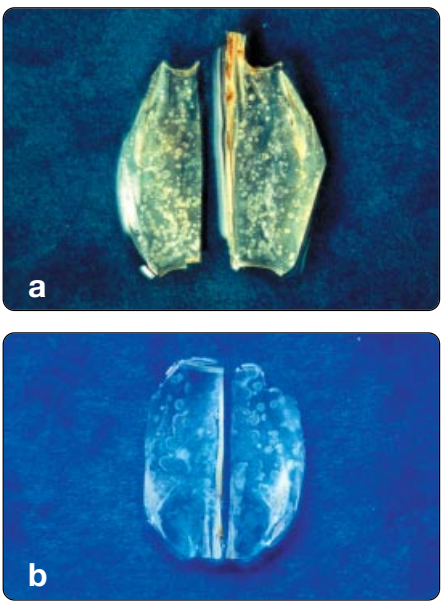

Fig. C.4a.4.2.2a, b. Bacterial white spots (BWS), which are less dense than virus-induced white spots. Note some BWS have a distinct whitish marginal ring and maybe with or without a pinpoint whitish dot in the center

(M. Shariff/ Wang *et al.* 2000 (DAO 41:9-18))

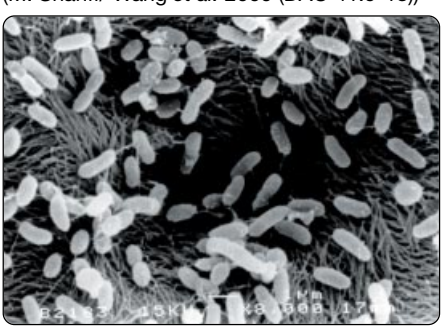

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Fig. C.4a.4.2.2c. Presence of large number of bacteria attached to exposed fibrillar laminae of the endocuticle.

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 pondwater pH (see C.4.6).

C.4a Bacterial White Spot Syndrome (BWSS)

C.4a.7 **Selected References**

Wang, Y.G., M. Shariff, K.L. Lee and M.D. Hassan. 1999. A review on diseases of cultured shrimp in Malaysia. Paper was presented at Workshop on Thematic Review on Management Strategies for Major Diseases in Shrimp Aquaculture, 28-30 November 1999, Cebu, Philippines. WB, NACA, WWF and FAO.

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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)

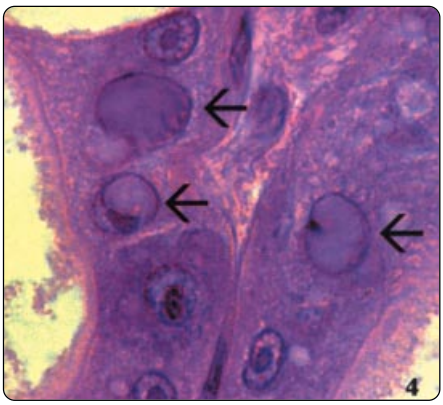
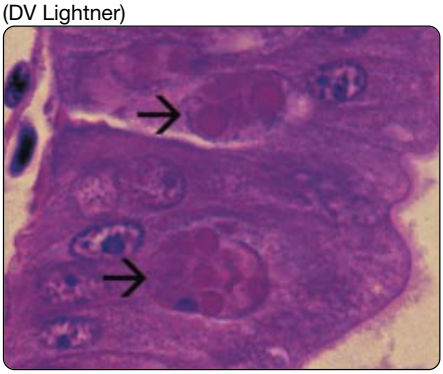


Fig.C.5.1.2a. Section of the hepatopancreas of *P. plebejus* displaying several hepatopancreas cells containing BMN-type intranuclear inclusion bodies. Mayer-Bennett H&E. 1700 x magnification.

(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)

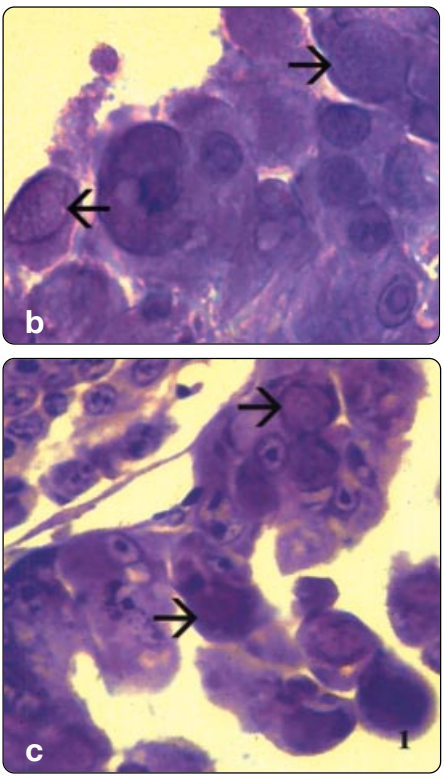


Fig. C.5.4.2.1b, c. Sections of the hepatopancreas of a PL of *P. japonicus* with severe BMN. Hepatopancreas tubules are mostly destroyed and the remaining tubule epithelial cells contain markedly hypertrophied nuclei that contain a single eosinophilic to pale basophilic, irregularly shaped inclusion 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)

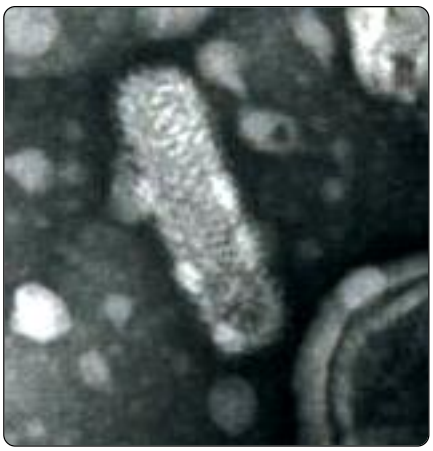

Fig. C.6.4.2.1. Transmission electron microscopy of GAV.

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').

C.7 Spawner-Isolated Mortality Virus Disease (SMVD)

(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.8.3.1.3 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)

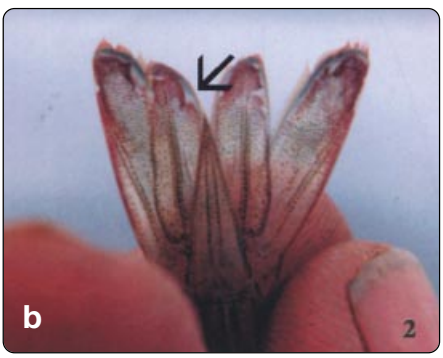

Fig. C.8.4.1.1a,b. a. Moribund, juvenile, pond-reared *Penaeus vannamei* from Ecuador in the peracute phase of Taura Syndrome (TS). Shrimp are lethargic, have soft shells and a distinct red tail fan; b. Higher magnification of tail fan showing reddish discoloration and rough edges of the cuticular epithelium 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)

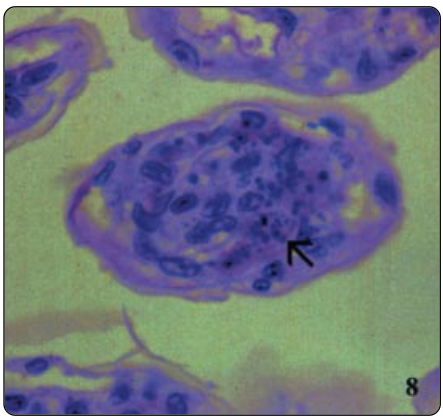

Fig. C.8.4.1.2a. Focal TSV lesions in the gills (arrow). Nuclear pykinosis and karyorrhexis, increased cytoplasmic eosinophilia, and an abundance of variably staining generally spherical cytoplasmic inclusions are distinguishing characteristics of the lesions. 900x magnification.

(DV Lightner)

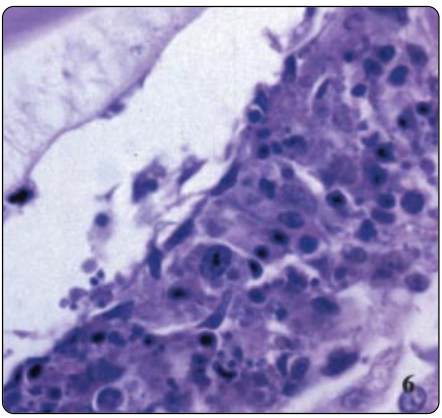

Fig. C.8.4.1.2c. Higher magnification of Fig. C.8.4.1.2b showing the cytoplasmic inclusions with pyknotic and karyorrhectic nuclei giving a 'peppered' appearance. Mayer-Bennett H&E. 900x magnification.

(DV Lightner)

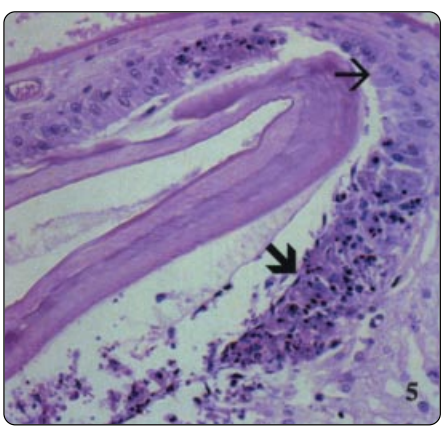

Fig. C.8.4.1.2b. Histological section through stomach of juvenile *P. vannamei* showing prominent areas of necrosis in the cuticular epithelium (large arrow). Adjacent to focal lesions are normal appearing epithelial cells (small arrows). Mayer-Bennett H&E. 300x magnification.

(DV Lightner)

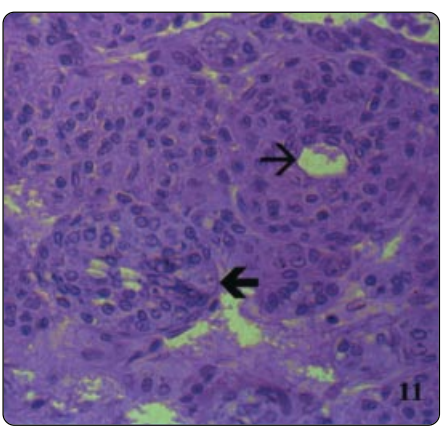

Fig. C.8.4.1.2d. Mid-sagittal section of the lymphoid organ (LO) of an experimentally infected juvenile *P. vannamei*. Interspersed among normal appearing lymphoid organ (LO) cords or tissue, which is characterized by multiple layers of sheath cells around a central hemolymph vessel (small arrow), are 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 subclinical 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 co-cultured species may also acquire MBV-type virus infections, but these have not been associated with severe pathology, or developed non-monodon 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)

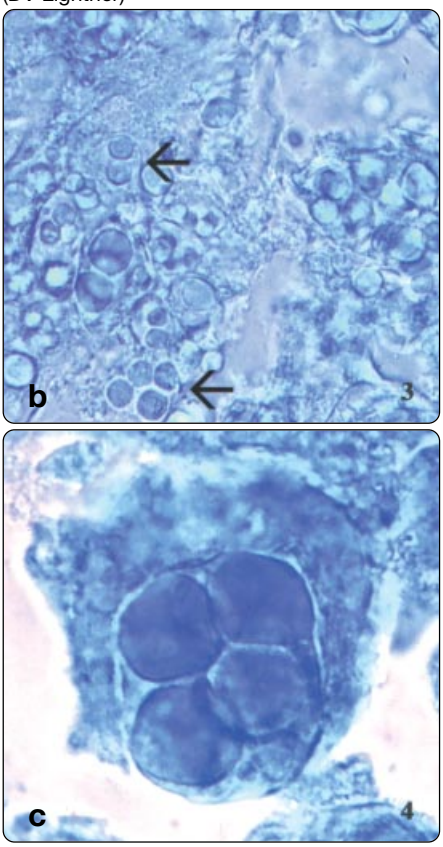
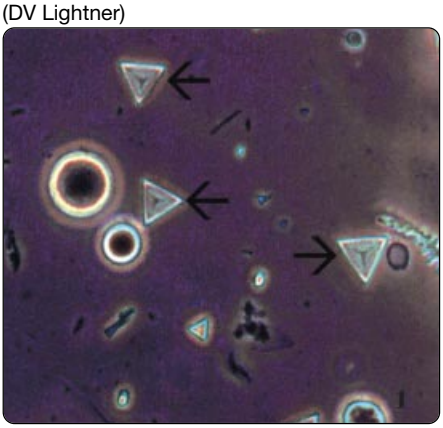


Fig. C.9.3.2.1b,c. Mid and high magnification views of tissue squash preparations of the hepatopancreas (HP) from PL of *P. monodon* with MBV infections. Most HP cells in both PLs usually display multiple, generally spherical, intranuclear occlusion bodies (arrow) that are diagnostic for MBV. 0.1% malachite green. 700x (b) and 1 700x (c) magnifications.

(DV Lightner)



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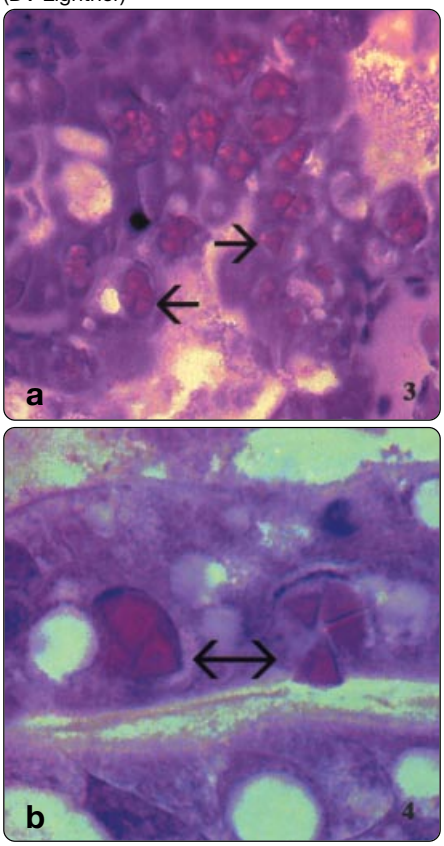

Fig. C.9.3.2.3a,b. a. Mid-magnification view of mid-sagittal sections of PL of *P. vannamei* with severe BP infections of the hepatopancreas showing multiple eosinophilic BP tetrahedral occlusion bodies within markedly hypertrophied hepatopnacreas (HP) cell nuclei (arrows). Mayer-Bennett H&E. 700x magnification; 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.

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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.

C.9 Nuclear Polyhedrosis Baculoviroses

(*Baculovirus penaei* [BP] PvSNPV; Monodon Baculovirus [MBV]PmSNPV)

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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C.9 Nuclear Polyhedrosis Baculoviroses

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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)

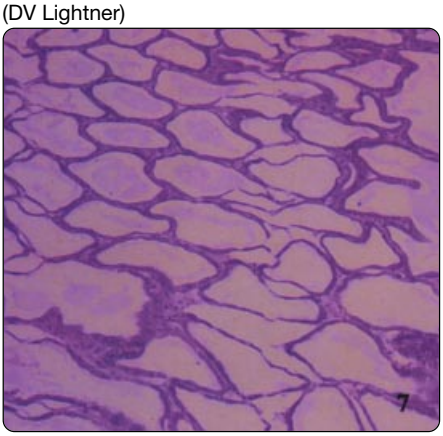
(DV Lightner)

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)



(DV Lightner)

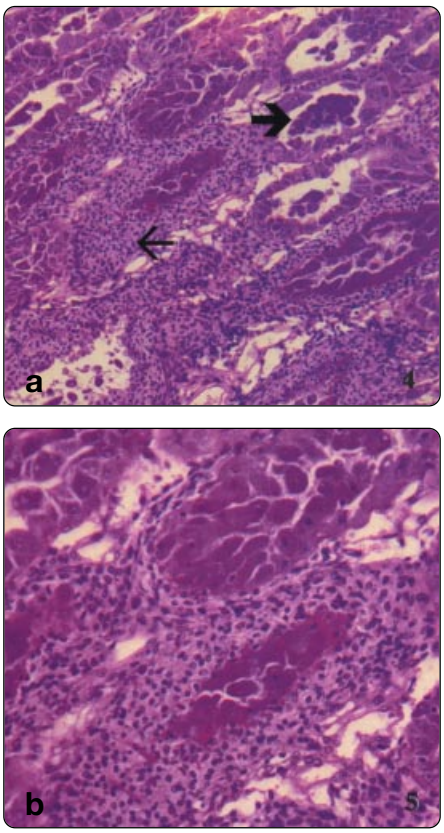


Fig. C.10.4.1.3a,b. Low and mid-magnification of photographs of the HP of a severely NHP infected juvenile *P. vannamei*. Severe hemocytic inflammation of the intratubular spaces (small arrow) in response to necrosis, cytolysis and sloughing of HP tubule epithelial cells (large arrow), are among the principal histopathological changes due to NHP. Mayer-Bennett H&E. 150x (a) and 300x (b) magnifications.

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

C.10 Necrotising Hepatopancreatitis (NHP)

(DV Lightner)

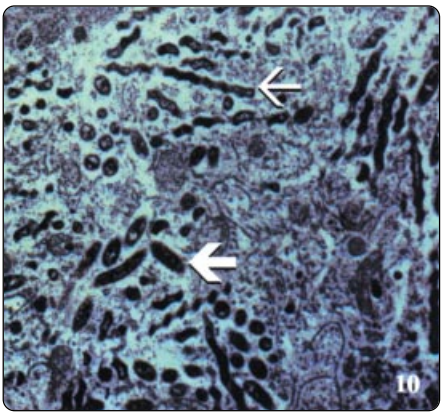


Fig. C.10.4.1.3d. The HP tubule epithelial cells show no cytoplasmic lipid droplets, but instead contain masses of the tiny, non-membrane bound, intracytoplasmic NHP bacteria (arrow). Mayer-Bennett H&E. 1700x magnification.

(DV Lightner)

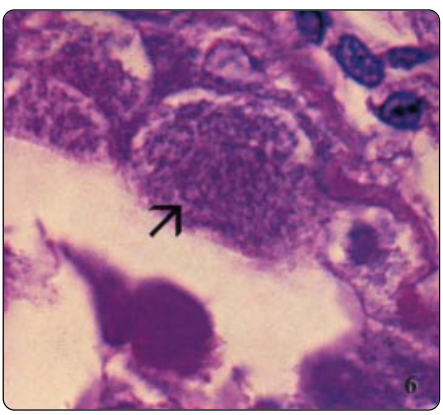


Fig. C.10.4.2.1. Low magnification TEM of a hepatopancreatocyte from a juvenile *P. vannamei* with NHP. Profiles of intracellular rod-shaped forms (large arrow) and helical forms (small arrow) of the NHP bacterium are abundant in the cytoplasm. 10 000x magnification.

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 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):

- 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)

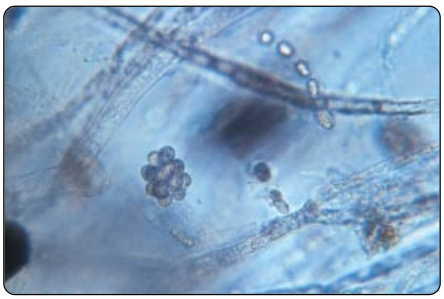

Fig. C.11.3.2.1a. Fresh microscopic mount of a piece of infected exoskeleton showing fungal spores.

(EAFP/DJ Alderman)

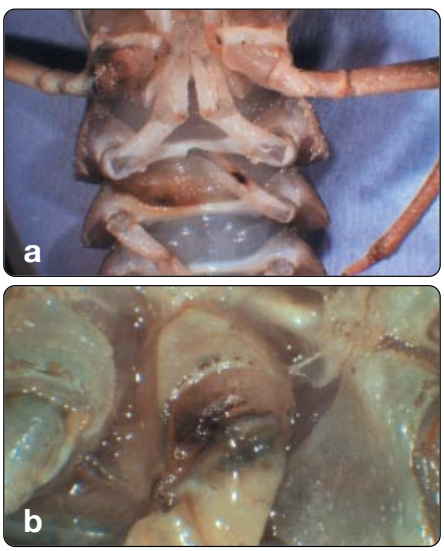

Fig. C.11.4.1.1a,b. Clinical signs of infected crayfish showing whitened necrotic musculature in the tail, and often accompanied in chronic infections by melanisation (blackening) of affected exoskeleton.

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
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ANNEX C.AII. LIST OF REGIONAL RESOURCE EXPERTS FOR CRUSTACEAN DISEASES IN ASIA-PACIFIC[1]

Disease	Expert
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	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
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	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
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	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
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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
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