CHAPTER 2.2.11.

TETRAHEDRAL BACULOVIROSIS (*BACULOVIRUS PENAEI*)

1. Scope[1]

For the purpose of this chapter, tetrahedral baculovirosis is considered to be infection with *Baculovirus penaei*. Synonyms: PvSNPV (singly enveloped nucleopolyhedrovirus from *Penaeus vannamei*).

2. Disease information

2.1. Agent factors

Aetiological agent: *Baculovirus penaei* (BP) as described by Couch (Couch, 1974; Couch, 1991), Summers, 1977, Overstreet, 1994and Bonami et al., 1995.

Fauquet et al., 2005in The International Committee on Virus Taxonomy, Eighth Report lists the related virus MBV (spherical baculovirosis) as a tentative species in the genus *Nucleopolyherdovirus*. Therefore, BP should also be considered as a tentative species in this genus.

2.1.1. Aetiological agent, agent strains

Agent strains: at least three geographical strains have been demonstrated. These are: 1) south-east Atlantic and Gulf of Mexico coasts of the USA and the Caribbean; 2) Pacific Coast of South, Central and North America; and 3) Hawaii (Brock et al., 1986; Bruce et al., 1993; Durand et al., 1998).

2.1.2. Survival outside the host

No data.

2.1.3. Stability of the agent (effective inactivation methods)

No data.

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species

BP infections have been reported in one or more species of the following penaeid genera or subgenera (the latter are between brackets): *Penaeus* (*Litopenaeus*), (*Farfantepenaeus*), (*Fenneropenaeus*), (*Melicertus*), (*Penaeus*), *Trachypenaeus* and *Protrachypene* (Bueno et al., 1990; Durand et al., 1998; Le Blanc et al., 1991; Lightner, 1996; Lightner et al., 1989; Machado et al., 1995; Overstreet, 1994). All penaeid species may be potential hosts (Lightner, 1996; Lightner, 1999; Overstreet, 1994).

2.2.2. Susceptible stages of the host

Susceptible stages of the host: all life stages, except eggs and nauplii, are susceptible to infection by BP.

1 NB: Version adopted by the World Assembly of Delegates of the OIE in May 2012. This disease is no longer listed by the OIE.

2.2.3. Species or subpopulation predilection (probability of detection)

No data.

2.2.4. Target organs and infected tissue

BP is strictly enteric infecting mucosal epithelial cells of the hepatopancreas tubules and the anterior midgut (Brock & Lightner, 1990; Couch, 1974; Couch, 1991; Johnson & Lightner, 1988; Lightner, 1996).

2.2.5. Persistent infection with lifelong carriers

Persistent infection occurs commonly in penaeid hosts of BP. Wild adult *P. vannamei* females that are heavily infected with BP have been shown to excrete BP-contaminated faeces when spawning, thereby contaminating the eggs and passing the virus to the next generation (Johnson & Lightner, 1988; Lightner, 1996).

2.2.6. Vectors

None are known in natural infections, but the rotifer *Brachionus plicatilis* and *Artemia salina* nauplii were used as passive carriers of BP to deliver the virus to larval stages of *P. vannamei* in experimental infections (Overstreet et al., 1988; Stuck & Wang, 1996).

2.2.7. Known or suspected wild aquatic animal carriers

None has been demonstrated.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission of BP is horizontal by ingestion of infected tissue (cannibalism), faeces, occlusion bodies, or contaminated detritus or water (Johnson & Lightner, 1988; Lightner, 1996; Overstreet et al., 1988).

2.3.2. Prevalence

Highly variable, from <1% in wild and cultured populations up to 100% in cultured populations in larval rearing tanks and nursery ponds (Brock & Lightner, 1990; Lightner, 1996).

2.3.3. Geographical distribution

BP is enzootic in wild penaeids in the Americas and Hawaii. It has not been reported in wild or cultured penaeid shrimp in the eastern hemisphere despite numerous introductions of American penaeids to Asia and the Indo-Pacific (Bondad-Reantaso et al., 2001; Brock et al., 1986; Lightner, 1996).

2.3.4. Mortality and morbidity

The larval stages (specifically protozoea and mysis) and early postlarval (PL) stages are the most easily infected in laboratory challenge studies (Bruce et al., 1994; Hammer et al., 1998; Le Blanc & Overstreet, 1990; Overstreet et al., 1988; Stuck & Overstreet, 1994; Stuck et al., 1996; Stuck & Wang, 1996) and are the stages where the highest mortalities are likely to occur in penaeid shrimp hatcheries. High mortality rates are unusual as a consequence of BP infection in the juvenile or adult stages, but infection may cause poor growth and reduced survival in nursery or grow-out ponds at shrimp farms (Brock & Main, 1994; Lightner, 1996; Overstreet, 1994).

2.3.5. Environmental factors

No data.

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccination methods for BP have been developed.

2.4.2. Chemotherapy

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3. Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4. Resistance breeding

The potential for selective breeding for BP resistance has been demonstrated.

2.4.5. Restocking with resistant species

Not applicable to BP.

2.4.6. Blocking agents

Blocking agents have not been reported.

2.4.7. Disinfection of eggs and larvae

Inactivation of BP by disinfectants, low pH, heat and UV irradiation has been reported (Le Blanc & Overstreet, 1991).

2.4.8. General husbandry practices

2.4.8.1. Hatchery

A number of husbandry practices have been applied for the prevention of BP infections and disease. Prescreening of broodstock for BP has been somewhat effective in detecting heavily infected carriers of the virus and thereby reducing the transmission of the disease from parent to offspring. With nonlethal testing methods, this is accomplished by simple light microscopic examination of faecal strands (or by polymerase chain reaction [PCR] testing of faecal strands if PCR testing facilities are readily available). Alternatively, spent broodstock may be killed after spawning and simple light microscopic examination of a hepatopancreas squash can be run (or the excised hepatopancreas may be tested by PCR) to determine the spawner's BP infection status. Because BP is transmitted from adults to their offspring by faecal contamination of the spawned eggs, prevention of infection in hatcheries may be achieved by taking additional steps to eliminate faecal contamination of spawned eggs and larvae by thoroughly washing nauplii or eggs with formalin, iodophores, and clean sea water (Chen et al., 1990). The routine disinfection of spawned eggs from infected or potentially infected broodstock has reduced the incidence of BP epizootics in hatcheries (Lightner & Redman, 1998).

2.4.8.2. Nursery and grow-out ponds

BP infections remain common in earthen-bottom ponds in regions of the Americas where the virus is enzootic (Bondad-Reantaso et al., 2001; Lightner, 1996), but incidence and prevalence of BP infections may be reduced in lined nursery and grow-out ponds.

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection by BP using molecular methods (e.g. PCR, *in-situ* hybridisation, etc.) include postlarvae (PL), juveniles and adults, when enteric tissues or organs are present. While BP may infect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in the larval stages, so these life stages may not be suitable samples for BP detection or certification for BP disease freedom.

3.2. Preservation of samples for submission

For routine histology or molecular assays, and guidance on preservation of samples for the intended test method see Chapter 2.2.0.

3.3. Pooling of samples

Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, subadults and adults. However, for eggs, larvae and postlarvae (PL), pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs or tissues

BP is an enteric virus and can be detected in the hepatopanceas.

Faecal samples can be collected when non-lethal testing is required (e.g. for non-lethal testing of valuable broodstock).

3.5. Samples/tissues that are not suitable

BP is an enteric virus (e.g. the hepatopancreas, the midgut, or its caeca) and does not replicate systemically.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

See Section 4.2for a description of gross clinical signs presented by shrimp infected with BP.

4.1.1.1. Direct microscopic examination

4.1.1.1.1. Wet mounts of fresh tissue

Diagnosis of BP infections is made by the demonstration of single or multiple tetrahedral occlusion bodies in epithelial cell nuclei in squash preparations of hepatopancreas or midgut examined by phase-contrast or bright-field microscopy. Occlusion bodies are tetrahedral or pyramidal in three-dimensional form, and range in size from less than 0.1 µm to nearly 20 µm from pyramidal base to peak, with a modal, vertical length of 8 µm. In some publications, the occlusion bodies of BP are referred to as PIBs (polyhedral inclusion bodies) (Bondad-Reantaso et al., 2001; Brock & Main, 1994; Lightner, 1996).

4.1.1.1.2. Wet mounts of faecal strands

This nonlethal method may be used to screen for carriers of BP. The method can be applied to juvenile or older shrimp, and it is perhaps most useful as a nonlethal method for screening valuable broodstock. Faecal samples from shrimp to be tested may be obtained by placing the shrimp in an aquarium, spawning tank, or other suitable tanks for a few hours until faecal strands are present on the tank bottom. The faecal strands are best collected using a clear plastic siphon hose (an airline fitted with a section of plastic pipette as a tip is ideal) and placed in a beaker, cup, or suitable container. The faecal strands may be made into wet mounts and examined directly for occlusion bodies. BP occlusion bodies are prominent, refractive tetrahedrons that range from just resolvable to nearly 20 µm in height (Bondad-Reantaso et al., 2001; Brock & Main, 1994; Lightner, 1996).

4.1.1.1.3. Collected faeces

Collected faeces may also be used as the sample for nonlethal testing for BP by PCR. PCR will provide greater diagnostic sensitivity for low-grade infections than will direct microscopic examination (Bondad-Reantaso et al., 2001; Lightner & Redman, 1998).

4.1.2. Behavioural changes

Other than lethargy in severely affected PLs, no behavioural changes in infected hosts have been reported.

4.2. Clinical methods

4.2.1. Gross pathology

Protozoea, mysis and early PL stages with severe BP infections may present a whitish midgut (because of the presence of occlusion bodies and cell debris in the faecal material). Juveniles and adults present no gross signs of diagnostic value, nor do larvae with less severe infections.

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

See Section 4.2.6

4.2.4. Wet mounts

See Section 4.1.1

4.2.5. Smears

Not applicable.

4.2.6. Fixed sections

4.2.6.1. Histopathology

Histology may be used to provide a definitive diagnosis of BP infection. Because 10% buffered formalin and other fixatives provide, at best, only fair fixation of the shrimp hepatopancreas (the principal target organ for BP and other baculovirus infections of penaeid shrimp), the use of Davidson's fixative (containing 33% ethyl alcohol [95%], 20% formalin [approximately 37% formaldehyde], 11.5% glacial acetic acid, and 33.5% distilled or tap water) is highly recommended for all routine histological studies of shrimp (Bondad-Reantaso et al., 2001; Lightner & Redman, 1998). Routine histological stains such as Mayer-Bennett's or Harris' haematoxylin and eosin (H&E) may be used for the demonstration of pathognomonic (for BP) tetrahedral occlusion bodies in hepatopancreatocytes, gut epithelial cells, or gut lumen (Bonami et al., 1995; Bondad-Reantaso et al., 2001; Lightner, 1996). Typically, BP-infected hepatopancreatic (or occasionally midgut) cells will present markedly hypertrophied nuclei with single or, more often, multiple eosinophilic occlusion bodies, chromatin diminution and margination (Bondad-Reantaso et al., 2001; Lightner, 1996). Occlusion bodies may be stained bright red with H&E stains, and intensely, but variably, with Gram's tissue stains. For example, Brown and Brenn's histological Gram stain, although not specific for baculovirus occlusion bodies, tends to stain occlusions more intensely (either red or purple, depending on section thickness, time of decolourising, etc.) than the surrounding tissue, which may aid in demonstrating their presence in low-grade infections (Bondad-Reantaso et al., 2001; Brock & Lightner, 1990; Lightner, 1996).

4.2.6.2. Autofluorescence method with phloxine stain

Another method for detecting BP occlusion bodies is based on the fluorescence of phloxine-stained occlusion bodies. Aqueous 0.001% phloxine may be added to tissue squash preparations to make wet-mounts of hepatopancreas or faeces for direct examination. Histological sections stained with routine H&E containing 0.005% phloxine, are also suitable for this procedure. BP occlusions in wet mounts of tissue squashes, in faeces, or in histological sections fluoresce bright yellow–green against a pale green background under epi-fluorescence (barrier filter of 0–515 nm and a 490 nm exciter filter). Other objects in the tissues and insect baculovirus occlusion bodies do not fluoresce with this method. Hence, the method can provide a rapid and specific diagnosis (Bondad-Reantaso et al., 2001; Lightner, 1996).

4.2.6.3. *In-situ* hybridisation

See Section 4.3.1.2.3below.

4.2.7. Electron microscopy/cytopathology

BP infection can be confirmed by demonstration of the virus (or pathognomonic occlusion bodies) in sections, or demonstration of the virus in semi-purified virus preparation prepared from the hepatopancreas (Couch, 1974; Couch, 1991; Johnson & Lightner, 1988).

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.1.1

4.3.1.1.2. Smears

See Section 4.1.2

4.3.1.1.3. Fixed sections

See Section 4.2.6

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

None reported to date.

4.3.1.2.2. Antibody-based antigen detection methods

Antibody-based methods: polyclonal antibodies for detection of BP have been developed and reported (Lightner, 1996), but none is available for routine diagnosis of BP infections.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. In-situ hybridisation

The *in-situ* hybridisation method using a DIG-labelled DNA probe for BP follows generally the methods outlined in Poulos et al., 1994and Lightner, 1996and given below.

i) Embed Davidson's fixed tissue in paraffin and cut sections at 4 µm or less thickness. Place sections on to positively charged microscope slides. Do not use gelatin in water to float sections; use only distilled or de-ionised water.

ii) Heat the slides for 30–45 minutes at 60°C. Rehydrate the tissue as follows:

Xylene (or suitable substitute)	3 ×	5 minutes each
Absolute alcohol	2 ×	1 minute each
95% alcohol	2 ×	10 dips each
80% alcohol	2 ×	10 dips each
50% alcohol	1 ×	10 dips each
Distilled water	6 ×	10 dips each (do not let slides dry out)

iii) Pipette 500 µl of 100 g ml^{-1} proteinase K prepared fresh in TNE buffer. Incubate for 15 minutes at 37°C.

iv) Wash slides in cold 0.4% formaldehyde for 5 minutes.

v) Wash slides in 2 × SSC for 5 minutes at room temperature.

vi) Prehybridise slides using 500 µl of hybridisation buffer and incubate for 30 minutes at 42°C in hybridisation chamber.

vii) Dilute DIG-labelled specific probe in hybridisation buffer to appropriate concentration and boil for 10 minutes. Quench on ice for 5 minutes.

viii) Pipette 500 µl of probe on to slide. Place on 85°C heat block for 6–7 minutes. Quench slides on ice for 5 minutes. Incubate overnight at 42°C in hybridisation chamber.

ix) Wash slides as follows:

2 × SSC	2 ×	15 minutes room temperature
1 × SSC	2 ×	5 minutes at 42°C
0.5 × SSC	2 ×	15 minutes at 42°C
Buffer I	1 ×	15 minutes room temperature

x) Pipette 500 µl Buffer II (Blocking Buffer). Incubate at 37°C for 30 minutes.

xi) Pipette 250 µl of anti-DIG-AP antibody (dilute 1 µl in 1 ml Buffer II). Incubate at 37°C for 30 minutes.

xii) Wash slides as follows:

Buffer I	2 ×	10 minutes room temperature
Buffer III	1 ×	5 minutes room temperature

xiii) Mix 4.5 µl NBT (nitroblue tetrazolium) and 3.5 µl X-phosphate (bromochloro-indoyl phosphate) for each 1 ml of Buffer III containing 1% polyvinyl alcohol. Pipette 500 µl onto each slide and incubate for 1-3 hours in the dark at room temperature in a humid chamber.

xiv) Stop the colour reaction in Buffer IV for 5 minutes at room temperature.

xv) Counter-stain and dehydrate the slides as follows:

Distilled water	1 ×	10 dips
0.5% Bismarck Brown	1 ×	2–5 minutes
95% alcohol	3 ×	10 dips each
Absolute alcohol	3 ×	10 dips each
Xylene (or suitable substitute)	4 ×	10 dips each

xvi) Mount with Permount and a cover-slip. Examine for cell-associated purple/black precipitate.

NOTES:

- This protocol can be performed in an incubator designed for *in-situ* hybridisation or a food dehydrator can be used as the heat source for the *in-situ* incubation chamber. Slides can be placed into pipette boxes, with water in the bottom, closed and placed in the dehydrator. It is important to have a temperature control and a humid atmosphere.

- The hybridisation step (viii) can be performed using a cover-slip to reduce evaporation. If a cover-slip is used, the volume of probe can be reduced to 250 µl.

- The heating step at 85°C is necessary to denature the double-stranded DNA genome. If reactions do not develop, inadequate heating to denature the genome is the most likely reason.

- Proteinase K is needed to eliminate protein bound to nucleic acid and to increase probe binding to nucleic acid.

- See the Reagents Section (below) for formulas of buffers used in this procedure.

4.3.1.2.3.1.1. Reagent and buffers for in-situ hybridisation method

i) 10 × Tris/NaCl/EDTA (ethylene diamine tetra-acetic acid) (TNE) buffer

0.5 Tris base	60.57 g
100 mM NaCl	5.84 g
1 mM EDTA-2 H_2O	3.72 g
DD H_2O	900 ml

pH to 7.4 with concentrated or 5 M HCl. QS to 1 litre. Autoclave. Store at 4°C.
To make 1 × TNE, dilute 100 ml 10 × stock in 900 ml DD H_2O.
Filter 1 × solution through 0.45 µm filter.

ii) Lysozyme, 100 µg ml^{-1} (prepare just prior to use)

1 × TNE	10 ml
Lysozyme	1 mg

iii) Proteinase K, 100 µg ml^{-1} (prepare just prior to use)

1 × TNE	10 ml
Proteinase K	100 µl stock proteinase K (10 mg ml^{-1})

Stock PK: add 100 mg proteinase K to 10 ml DDH_2O. Dispense 100 µl into tubes and store at –20°C. Prepare working concentration (100 µg ml^{-1}) just prior to use.

iv) 0.4% formaldehyde

37% formaldehyde	5.4 ml
DD H_2O	500 ml

Store at 4°C. It can be reused five times or stored for 3 months before discarding.

v) Hybridisation buffer (50 ml final volume)

4 × SSC	10 ml 20 × SSC
50% formamide	25 ml 100% formamide
1 × Denhardt's	2.5 ml 20 × Denhardt's
0.5 mg ml^{-1} salmon sperm DNA	2.5 ml of 10 mg ml^{-1} solution
5% dextran sulfate	10 ml 25% dextran sulfate

Store at 4°C.

vi) 20 × SSC buffer

3 M NaCl	175.32 g
0.3 M Na citrate-2H_2O	88.23 g
DD H_2O	QS to 1000 ml

pH to 7.0. Autoclave. Store at 4°C.
To make 2 × SSC, dilute 100 ml 20 × SSC in 900 ml DD H_2O. To make 1 × SSC, dilute 50 ml 20 × SSC in 950 ml DD H_2O. To make 0.5 × SSC, dilute 50 ml 20 × SSC in 1950 ml DD H_2O.
Filter solutions through 0.45 µm filter. Store at 4°C.

vii) 20 × Denhardt's solution

BSA (Fraction V)	0.4 g bovine serum albumin
Ficoll 400	0.4 g Ficoll
PVP 360	0.4 g polyvinylpyrollidine
DD H_2O	100 ml

Filter through 0.45 µm filter. Store at 4°C.

viii) 25% dextran sulfate

Dextran sulfate	25 g
DD H_2O	QS to 100 ml

Warm on low heat with stirring until dissolved. Store frozen.

ix) Salmon sperm DNA (10 mg ml^{-1})

Salmon sperm DNA	0.25 g
DD H_2O	25 ml

Slowly add DNA to water in a beaker with a stir bar. Heat and stir to dissolve DNA, adding more until all the DNA is dissolved. Autoclave. Dispense into sterile tubes. Store at –20°C.

x) 10 × Buffer I

1 M Tris base	121.1 g
1.5 M NaCl	87.7 g
DD H_2O	QS to 1000 ml

pH to 7.5 with HCl. Autoclave. Store at 4°C.
To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml DD H_2O.
Filter through 0.45 µm filter. Store at 4°C.

xi) Buffer II (blocking buffer and Ab Dilution Buffer)

Genius reagent 11	0.5 g
Buffer I	100 ml

Warm on low heat with stirring 30 minutes to dissolve. Solution will be cloudy, but with no particulates. Store at 4°C for up to 2 weeks.

xii) Buffer III

100 mM Tris base	12.11 g
100 mM NaCl	0.58 g
DD H_2O	QS to 1000 ml

pH to 9.5 with HCl.
Then add:

50 mM $MgCl_2.6H_2O$	16.10 g

Filter through 0.45 µm filter. Store at 4°C.

xiii) 10% polyvinyl alcohol (PVA)

Polyvinyl alcohol (30,000-70,000 MW)	10 g
DD H_2O	100 ml

Stir PVA and warm, if necessary, to get into solution. Dispense 10 ml/tube. Store at –20°C.

xiv) Development solution

Mix 90 ml Buffer III with 10 ml of 10% PVA and store at 4°C. Just prior to use, for each 1 ml of Buffer III with PVA add:

Nitroblue tetrazolium salt	4.5 µl NBT (75 mg ml^{-1} in 70% dimethylformamide)
5-bromo-4-chloro-3-indoyl phosphate, toluidinum salt	3.5 µl X-phosphate (50 mg ml^{-1} in dimethylformamide)

xv) 10 × Buffer IV

10 mM Tris base	1.21 g
1 mM EDTA.$2H_2O$	3.7 g
DD H_2O	QS to 1000 ml

pH to 8.0 with 5 N HCl. Autoclave. Store at 4°C.
To make 1 × Buffer IV, dilute 100 ml of 10 × stock in 900 ml DD H_2O.
Filter through 0.45 µm filter. Store at 4°C.

xvi) 0.5% Bismarck Brown Y

Bismarck Brown Y	2.5 g
DD H_2O	500 ml

Dissolve the stain in water. Filter through a Whatman No. 1 filter. Store at room temperature.

4.3.1.2.3.2. Polymerase chain reaction methods

The protocol described here is modified from Wang et al., 1996.

4.3.1.2.3.2.1. Appropriate samples

Appropriate samples are excised hepatopancreata, whole larvae or PLs (pooled), or faeces. Samples may be fresh, frozen, preserved in 90% ethanol, or other media designed for preservation of samples for DNA amplification.

Substances in the hepatopancreas and faeces of shrimp have been found to inhibit the DNA polymerase used in the PCR assay. Therefore, DNA extraction is required before PCR can be successfully used to detect BP.

4.3.1.2.3.2.2. DNA extraction

DNA extraction kits are convenient and commercially available. Otherwise, a suitable DNA extraction procedure is as follows:

i) A sample of faeces or hepatopancreas is added to digestion buffer (~1:10 ratio of sample:buffer in up to 400 µl of buffer) containing 50 mM KCl, 10 mM Tris/HCl, pH 8.3, 0.1 mg ml^{-1} gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 80 µg ml^{-1} proteinase K, crushed, and dispersed with a wooden toothpick or a pipette tip.

ii) After dispersing the sample in the digestion buffer, heat to 60°C for 1 hour and then to 95°C for 10 minutes.

iii) Centrifuge at 12,000 ***g*** for 2 minutes, transfer the supernatant fluid to a new tube, and store on ice.

iv) Remove 50 µl of the digested sample and dilute with 150 µl of dilution buffer (10 mM Tris/HCl, pH 8.0, 0.1 mM EDTA) and extract with 200 µl of phenol/isoamyl alcohol/chloroform (PIC) (25/1/24).

v) After vortexing the sample for 5 seconds, leave the tube for 5 minutes and then centrifuge at 12,000 ***g*** for 2 minutes.

vi) Remove 160 µl of the aqueous (upper) phase and transfer to a new microcentrifuge tube.

vii) The extraction step may be repeated if necessary.

viii) Precipitate the DNA by adding 20 µg of glycogen (1 µl of a 20 mg ml^{-1} stock), 65 µl of a 7.5 M ammonium acetate and 390 µl of ethanol, store at –20°C for >1 hour, and then pellet the DNA by centrifugation at 12,000 ***g*** for 5 minutes.

ix) Rinse the DNA pellet with 200 µl of 70% ethanol to remove residual ammonium acetate, dry, and then dissolve the DNA pellet in 30 µl of dilution buffer or distilled water prior to adding the sample (template) to the PCR reaction mixture and beginning the PCR.

4.3.1.2.3.2.3. PCR method (Wang et al., 1996)

4.3.1.2.3.2.3.1. Primers

Three forward and three reverse primers selected from a ~1430 base pair (bp) segment of the BP polyhedrin gene have been reported by Wang et al., 1996. The sequences for these primers are:

Primer	Sequence	Temperature
BPA	5'-GAT-CTG-CAA-GAG-GAC-AAA-CC-3'	61°C
BPB	5'-ATC-GCT-AAG-CTC-TGG-CAT-CC-3'	64°C

Primer	Sequence	Temperature
BPD	5'-TGT-TCT-CAG-CCA-ATA-CAT-CG-3'	62°C
BPE	5'-TAC-ATC-TTG-GAT-GCC-TCT-GC-3'	63°C
BPF	5'-TAC-CCT-GCA-TTC-CTT-GTC-GC-3'	68°C
BPG	5'-ATC-CTG-TTT-CCA-AGC-TCT-GC-3'	64°C

The combinations of these primers amplify segments from BP template DNA of: BPA/BPF – 196 bp; BPA/BPB – 560 bp; BPA/BPG – 933 bp; BPD/BPB – 207 bp; BPD/BPG – 580 bp; and BPE/BPG – 221 bp.

4.3.1.2.3.2.3.2. Procedure

The following PCR procedure was adapted from Wang et al., 1996:

i) For BP PCR, the DNA in each extracted sample is denatured by heating in a boiling water bath for 3 minutes followed by quick chilling in ice-water.

ii) 25 µl reaction mixture containing 5 mM of each primer, 1.5 mM $MgCl_2$, and 0.5-1 unit of DNA polymerase is added.

iii) After heating the reaction mixture for 3 minutes at 95°C, 30 PCR cycles (a DNA melting step at 94°C, a primer annealing step at 60°C, and an elongation step at 72°C) are performed followed by an elongation step of 5 minutes at 72°C.

iv) The resultant PCR products may be compared with molecular standards by 2% agarose gel electrophoresis or assayed for with a specific DNA probe for the fragment following a Southern transfer.

v) The following controls should be included in every PCR assay for BP: a known negative tissue or faecal sample; a known positive tissue or faecal sample (this can be the DNA clone from which a specific set of primers was designed); and a 'no- template' control.

4.3.1.2.3.2.4. Alternative PCR method

Alternative method used by the OIE Reference Laboratory at the University of Arizona (unpublished). Use sample type and extraction methods as described above.

4.3.1.2.3.2.4.1. Primers

One forward and reverse primer pair (6581F/6582R) selected from clone IR36 (referred to as B1.23 in Bonami et al., 1995and deposited in GenBank with accession number DQ496179) produces a 644 bp amplicon. The sequences for these primers are:

Primer	Sequence
6581	5'-TGT-AGC-AGC-AGA-GAA-GAG-3'
6582	5'-CAC-TAA-GCC-TAT-CTC-CAG-3'

4.3.1.2.3.2.4.2. Procedure

PCR reaction mixture:

Table 4.1.

Reagent	25 µl reaction mix	25 µl PCR beads[1]	Final conc.
dH_2O	16.5 µl	23.0 µl	–
10 × Buffer	2.5 µl	–	1 ×
dNTP's	0.5 µl each	–	200 µM each
Primer A	0.5 µl	0.5 µl	0.31 µM
Primer B	0.5 µl	0.5 µl	0.31 µM

Table 4.1.

Reagent	25 µl reaction mix	25 µl PCR beads[1]	Final conc.
$MgCl_2$	1.5 µl	–	1.5 µM
Enzyme	0.5 µl	–	2.5 U
Template	1.0 µl	1.0 µl	–

1. PuReTap™ Ready-To-Go PCR beads™, Amersham Biosciences, Buckinghamshire, UK

PCR cycling parameters:

Primers	Mix/Beads	Time	Temp. °C	No. cycles
6581/6582	Mix/Beads[1]	5 minutes	95	1
		30 seconds 30 seconds 1 minute	95 60 72	35
		7 minutes	72	1

4.3.1.2.4. Agent purification

None.

4.3.2. Serological methods

None applicable.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of BP are listed in Table 5.1.The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended and/or not available for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. *Tetrahedral baculovirosis (Baculovirus penaeid) surveillance, detection and diagnostic methods*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	c	d	d	d	d	d
Bioassay	d	d	d	d	c	c
Direct LM	b	b	c	c	a	a
Histopathology	b	b	c	c	a	a
Transmission EM	d	d	d	d	d	a
Antibody-based assays	d	d	d	c	d	d
***In situ*DNA probes**	c	c	c	c	a	a
PCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infection with Tetrahedral baculovirosis (*Baculovirus penaeid*)

Two years of history of negative test results for BP using:

- PCR performed on samples of the appropriate type and sample size; and/or
- Wet mount and/or histological results in which no tetrahedral occlusion bodies are observed in samples of the appropriate type and sample size.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

For larvae (especially protozoea, mysis and early PL stages): mortality with larvae presenting white midguts. For juveniles: poor growth in populations with a prior history of BP infection.

7.2. Definition of confirmed case

Any combination of at least two of the following three methods (with positive results):

- Microscopic demonstration of tetrahedral occlusion bodies in wet mounts of whole larvae or excised hepatopancreata. For older PLs, juveniles and adults: tetrahedral occlusion bodies evident in wet mount squashes and/or in histological sections of the hepatopancreas or faeces.
- *In-situ* hybridisation positive histological signal to BP-type lesions (i.e. hypertrophied nuclei with or without pathognomonic tetrahedral occlusion bodies).
- PCR positive results for BP.

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NB: There is not currently an OIE Reference Laboratory for Tetrahedral baculovirosis (*Baculovirus penaei*) (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/).