AQUATIC ANIMAL HEALTH STANDARDS COMMISSION
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Aquatic Manual

CHAPTER 2.4.1.

INFECTION WITH
ABALONE HERPES-LIKE VIRUS

1. Scope
For the purpose of this chapter, abalone viral ganglioneuritis is considered to be infection with abalone herpes-like virus (AbHV).

2. Disease information

2.1. Agent factors
Abalone herpes-like virus (AbHV) is the aetiological agent for abalone viral ganglioneuritis (AVG) a contagious viral disease of abalone in Australia (4, 5) and possibly abalone species in other countries (1, 8). However, whilst the relationship between the Australian viral isolate(s) and other herpes-like viral isolates has not, as yet, been elucidated, it is suggested that this virus is the second member of the Malacoherpesviridae along with Ostreid Herpesvirus-1 (3, 6).

2.1.1. Aetiological agent, agent strains
AbHV particles have been purified (7) and were observed by transmission electron microscopy to be icosahedral with electron dense cores and a diameter of 100–110 nm. The intranuclear location of the virus particles, their size and ultrastructure are characteristic of members of the Herpesviridae. Isopycnic gradient centrifugation (in potassium tartrate and caesium chloride gradients) indicated a buoyant density of 1.17–1.18 g/ml for the virus particles (7).

2.1.2. Survival outside the host
Not known – under investigation.

2.1.3. Stability of the agent (effective inactivation methods)
Under investigation.

2.1.4. Life cycle
Not applicable.

2.2. Host factors

2.2.1. Susceptible host species
Currently, species known to be susceptible to AVG in Australia are the greenlip abalone (Haliotis laevigata), blacklip abalone (H. rubra) and hybrids of these two species. Clinical signs consistent with AVG have not been reported in other molluscan species in areas where AVG is suspected to be enzootic. In Chinese Taipei, ganglioneuritis associated with a herpes-like viral infection and high mortalities in the abalone H. diversicolor supercxtexa has been reported. The disease was reported only in H. diversicolor supercxtexa, while cohabiting Japanese black abalone H. discus remained normal. (1). It
is not known whether the Australian virus is the same as, or different to, the virus found in Chinese
Taipei.

2.2.2. Susceptible stages of the host
all ages.

2.2.3. Species or subpopulation predilection (probability of detection)
No data.

2.2.4. Target organs and infected tissue
The major histopathological lesion identified in abalone affected with AVG is ganglioneuritis –
inflammation confined to neural tissue. The cerebral, pleuropedal and buccal ganglia can be affected as
well as the cerebral commissure and associated peripheral nerves (5).

2.2.5. Persistent infection with lifelong carriers
No data.

2.2.6. Vectors
No data.

2.2.7. Known or suspected wild aquatic animal carriers
No data

2.3. Disease pattern
Outbreaks of AVG in both farmed and wild abalone populations in Australia are associated with rapid onset of
high mortality rates (up to 90%) in all age classes. Similarly, in Chinese Taipei, during the epizootic in cultured
abalone (the water temperature was 16–19°C), both adult and juvenile abalone suffered from the disease, with
cumulative mortalities of 70–80%. It was reported that death of all of the abalone in a pond could occur within
3 days of the onset of clinical signs. A similar disease pattern occurred with experimental infections (1, 2).

2.3.1. Transmission mechanisms
Horizontal transmission (1, 2) has been demonstrated experimentally by:

1. exposing healthy abalone to water containing diseased abalone in the same tank without direct
   contact between the diseased and healthy abalone;
2. placing healthy abalone in water that was previously inhabited by diseased abalone; and
3. intramuscular injection of healthy abalone with a filtered tissue homogenate from diseased abalone.

In all cases, 100% mortality was observed with a preclinical period of 1–2 days following exposure and
then mortality commenced until 100% mortality occurred within 2–5 days post-infection.

2.3.2. Prevalence
In Victoria Australia, and similarly in Chinese Taipei, farms experiencing an outbreak of abalone viral
ganglioneuritis can expect a rapid rise in mortality rate (up to 90% or more). Affected abalone
demonstrating clinical signs (e.g. curling of the foot) are likely to die within 1 day of showing clinical
signs. Ganglioneuritis is observed in sections of neural tissue by light microscopy and confirmation of the
presence of abalone herpes-like virus is obtained by qPCR and/or *in-situ* hybridisation (2). Using these
methods there has been very few false positives or false negatives reported. The precise prevalence of
AVG in wild populations in Victorian waters is unknown.

2.3.3. Geographical distribution
Australia (Victoria and Tasmania), Chinese Taipei.
2.3.4. Mortality and morbidity
In on-farm epizootics in Victoria Australia cumulative mortality in all age classes can reach >90%. In experimental trials, 100% mortality can occur within 5 days post-exposure. The vast majority of abalone that display clinical signs are likely to die within 1–2 days.

2.3.5. Environmental factors
In Australia, the initial outbreak occurred on-farm in the summer months of 2005/2006 and subsequently appeared to spread to wild populations which experienced mortality throughout the following year i.e. during all seasons. All experimental infections to date have been carried out in artificial seawater in the temperature range 15–18°C. In Chinese Taipei, during the epizootic, the water temperature was 16–19°C, and experimental infections were carried out at 17–20°C. The precise temperature range for this virus is yet to be determined.

2.4. Control and prevention
In the absence of efficacious anti-viral agents, implementing high levels of on-farm biosecurity is recommended. Following an on-farm outbreak, destruction of infected stock, disinfection of water and equipment, and fallowing procedures appear to be effective at preventing reinfection. Prior to restocking the use of sentinel abalone can be used to test the status of the previously infected premises.

2.4.1. Vaccination
No vaccines available.

2.4.2. Chemotherapy
No data.

2.4.3. Immunostimulation
No data.

2.4.4. Resistance breeding
No data.

2.4.5. Restocking with resistant species
No data.

2.4.6. Blocking agents
No data.

2.4.7. Disinfection of eggs and larvae
No data.

2.4.8. General husbandry practices
To-date, experimental data indicates that the virus is highly virulent. Practices that could be implemented to reduce the severity of the disease have not been identified.

3. Sampling

3.1. Selection of individual specimens
At the first sign of an increase in weak or abnormally behaving abalone, or a sudden onset in mortality, live moribund individuals should be selected for sampling. If moribund or freshly dead abalone are not present, samples of healthy abalone from all parts of the farm and representing all age classes should be selected for sampling.
3.2. Preservation of samples for submission
Samples should be collected for examination by i) light microscopy (i.e. histology) and should be fixed in 10% formalin; ii) electron microscopy (fixed in 2.5% glutaraldehyde); iii) PCR (fixed in PCR preservative such as 95% ethanol). If fixatives are not available samples should be kept chilled (on ice) and forwarded to arrive at the laboratory within 24 hours. Alternatively, samples can be sent frozen (not suitable for histology or electron microscopy but can be used for PCR).

3.3. Pooling of samples
Fixed tissues can be pooled according to age class and pond/farm/geographical location.

3.4. Best organs or tissues
Neural tissue such as the cerebral, pleuropedal and buccal ganglia.

3.5. Samples/tissues that are not suitable
To date, lesions have not been detected consistently in non-neural tissues.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs
In Victoria Australia, AVG outbreaks in both farmed and wild populations were associated with high mortality rates (up to 90% on farm). Clinically, abalone may demonstrate one or more of the following signs: irregular peripheral concave elevation of the foot; swollen and protruding mouth parts; eversion of the radula; minimal movement of the pedal muscle; excessive mucus production; absence of the marked extension of the foot shown in the righting reflex when healthy abalone are turned onto their backs; reduced pedal adhesion to the substrate. In Tasmania, affected abalone observed in processing plants exhibited ‘hard foot’ or tetany; excessive mucus production; abnormal spawning; ‘bloating’ (4). Similar signs have been reported for an epizootic in Chinese Taipei (1).

4.1.2. Behavioural changes
AVG is an acute disease. Abalone are likely to die within 1–2 days of demonstrating clinical signs the disease.

4.2. Clinical methods

4.2.1. Gross pathology
Abalone that are loosely attached to the substrate due to abnormalities of the pedal muscle should be selected for sampling. If this gross pathology is due to acute AVG, it is likely that these abalone will die within 1-2 days.

4.2.2. Clinical chemistry
No data.

4.2.3. Microscopic pathology
Abalone affected with AVG demonstrate inflammation (increased infiltration by haemocytes) and necrosis confined to neural tissue (cerebral, pleuropedal and buccal ganglia, branches of the pedal nerve and peripheral nerves) as observed in histological sections of neural tissue stained with haematoxylin and eosin and examined by light microscopy (4, 5).
4.2.4. Wet mounts
Not applicable.

4.2.5. Fixed sections
_In-situ_ hybridisation localises AbHV-infected cells within the neural tissue which, on histological examination, demonstrates ganglioneuritis typified by an inflammatory change with increased cellularity involving mainly haemocytes and glial cells, and cell necrosis in the affected nerves.

4.2.6. Electron microscopy/cytopathology
Transmission electron microscopy can be used to confirm the presence of viral particles in infected ganglia. AbHV particles are icosahedral with electron dense cores and a diameter of 100–110 nm. The intranuclear location of the particles and their ultrastructure are characteristic of members of the Herpesviridae (7).

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods
Neural tissue (cerebral, pleuropedal and buccal ganglia, branches of the pedal nerve and peripheral nerves) is the prime target and should be sampled and fixed (using 10% formaldehyde and processed using standard procedures, and stained with haematoxylin and eosin) for histological examination.

Tissue samples (containing pleuropedal ganglion) for examination by electron microscopy should be fixed using 2.5% (v/v) glutaraldehyde and 2–4% (v/v) paraformaldehyde in 0.1 M cacodylate buffer and post-fixed in 1% (w/v) osmium tetroxide, washed in reverse osmosis water (3 × 5 minutes), dehydrated in a graded series of ‘analytical grade’ ethanol (70%, overnight at 4°C; 95%, 20 minutes; 100%, 3 × 20 minutes), infiltrated in 100% Spurr’s resin (overnight) and then embedded in Spurr’s resin.

4.3.1.1.1. Wet mounts
Not applicable.

4.3.1.1.2. Imprints
Not applicable.

4.3.1.1.3. Fixed sections
Neural tissue (cerebral, pleuropedal and buccal ganglia, branches of the pedal nerve and peripheral nerves) is the prime target and examination of histological sections reveals ganglioneuritis – increased cellularity involving mainly haemocytes and glial cells, and cell necrosis.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media
To date, attempts to culture the virus in both vertebrate and invertebrate cell lines have been unsuccessful.

4.3.1.2.2. Antibody-based antigen detection methods (IFAT, ELISA, etc.)
Not applicable.

4.3.1.2.3. Molecular techniques (PCR, ISH, sequencing, etc.)
Neural tissue samples should be fixed in preservative (80% reagent grade ethanol; 19.75% glycerol; 0.25% β-mercaptoethanol) or, alternatively, 95% ethanol.
4.3.1.2.3.1 Nucleic acid extraction

The pleuropedal ganglion and/or pedal nerve cords are dissected from the fixed tissue and placed in 1.5 ml tubes for DNA extraction. Nucleic acid from AbHV-infected and uninfected abalone tissues (approximately 20 mg of muscle and neural tissue) are extracted using a commercial kit, e.g. QIAamp DNA mini kit (QIAGEN) or equivalent, according to the manufacturer's instructions. Nucleic acid, bound to minicolumns, is eluted and resuspended in a final volume of 100 μl of buffer (~100 ng μl⁻¹) provided in the kit.

4.3.1.2.3.2 Conventional one-step PCR

Following DNA extraction, PCR amplification is performed on DNA samples extracted from tissues derived from i) positive (known AbHV-infected) control, ii) negative (known uninfected), and iii) test (submitted) abalone samples.

The PCR mixture for a single sample consists of the following reagents: 9.5 μl water; 12.5 μl HotStar Taq Master mix; 0.5 μl forward primer (20 μM); 0.5 μl reverse primer (20 μM); 2 μl DNA. The mixture is incubated in an automatic thermal cycler programmed with the following cycling conditions: one cycle at 94°C for 15 minutes; 35 cycles at 94°C for 15 seconds, 52°C for 30 seconds and 72°C for 30 seconds; and, finally, one cycle at 72°C for 5 minutes. Amplified DNA is detected following resolution of the amplicons by agarose (2%) gel electrophoresis.

Primers used:
Forward primer (007F): 5’-GCC-TTC-GCT-GGA-AGC-ATA-C-3’
Reverse primer (007R): 5’-GTG-GTC-GCG-AGA-AGA-GAA-C-3’

Interpretation
At the completion of the PCR, specific PCR fragments of the correct size (486 bp) are resolved by agarose gel electrophoresis:

- The negative control sample must have no evidence of specific amplified products.
- A positive control sample must yield a specific AbHV fragment (486 bp in size).
- Amplified fragments of the correct size are then extracted from the gel, and the DNA sequence is determined (by using the PCR primers as sequencing primers).
- Sequence identity is determined by sequence alignment.

4.3.1.2.3.3 TaqMan PCR assay

Following DNA extraction, a real-time PCR is carried out in a 96-well plate using 25 μl reaction volume containing 12.5 μl of TaqMan® Fast Universal PCR Master Mix (2×), 2 μl (~100 ng per μl) of extracted DNA sample and the reaction mixture is made up to 25 μl using deionised water after primers and probes are added at the appropriate concentrations. The following thermal cycling conditions are used: 95°C for 59 seconds followed by 45 cycles of 95°C for 3 seconds and 62°C for 30 seconds.

The AbHV primers and probe sequences are as follows:
Forward primer (ORF49F): 5’-AAC-CCA-CAC-CCA-ATT-TTT-GA-3’
Reverse primer (ORF49R): 5’-CCC-AAG-GCA-AGT-TTG-TTG-TT-3’
6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) labelled probe (ORF49Pr): 6FAM-CCG-CTT-TCA-ATC-TGA-TCC-GTAMRA.
The AbHV primers and probe are used at a final concentration of 300 nM and 100 nM, respectively.

18S ribosomal RNA gene primers and probe (Applied Biosystems) are used to validate the nucleic acid extraction procedure and the absence of PCR inhibitors. The 18S RNA gene endogenous control primers and probe sequences are as follows:
Forward primer (18S Forward) 5’-CGG-CTA-CCA-CAT-CCA-AGG-AA-3’
Reverse Primer (18S Reverse) 5’-GCT-GGA-ATT-ACC-GCG-GCT-3’
Probe (18S VIC – TAMRA probe) 5’-TGC-TGG-CAC-CAG-ACT-TGC-CCT-C-3’
Both the 18S RNA gene primers and the probe are used at a final concentration of 100 nM.

All samples (including positive and negative controls) are tested in duplicate or triplicate. The results of a TaqMan assay are expressed in the form of software-generated characteristic amplification curves. Amplification curves from positive and negative (no template controls) should be compared to the test sample. A sample is considered above the test background level when the change in fluorescence ($\Delta R_n$) of FAM or VIC, relative to that of ROX (internal reference dye), exceeds the threshold value which is arbitrarily set at the upper end of the linear range of the amplification plots. Results of a TaqMan assay can also be, and often are, expressed as cycle threshold ($C_T$) values. The cycle threshold ($C_T$) is defined as the cycle number at which a statistically significant increase in fluorescence output above background is detected.

At the completion of the TaqMan PCR assay, the presence of AbHV DNA is demonstrated by the presence of specific amplicons, identified by software-generated characteristic amplification curves and cycle threshold values ($C_T$). No-template controls must have no evidence of specific amplicons.

If the test is deemed valid, the results for the test sample wells may be interpreted using the following criteria:

- **Positive** test results are defined as the presence of specific amplicons expressed as a characteristic amplification curve similar to the positive control(s) and having a cycle threshold ($C_T$) value $<35.0$.
- **Negative** test results are defined as the absence of specific amplicons expressed by a characteristic amplification curve similar to the no-template control and having a cycle threshold ($C_T$) value equal to or greater than 36.0.

Indeterminate test results are defined as having a characteristic amplification curve similar to the positive control but a cycle threshold ($C_T$) value of 35.0–36.0. This necessitates repeating the assay with at least 3 test sample wells.

### 4.3.1.2.3.4 In-situ hybridisation

The in-situ hybridisation (ISH) procedure described here uses a digoxygenin (DIG)-labelled DNA probe to detect AbHV in formalin-fixed, paraffin-embedded (FFPE) tissue sections.

**Reagents**

- **20× SSC pH7** (store at room temperature)
  
  175.32 g litre$^{-1}$ NaNCl
  
  88.23 g litre$^{-1}$ Sodium citrate

- **100× Denhardt’s solution** (store at $-20^\circ$C)

- **2 g (100 ml)$^{-1}$** Bovine serum albumin (Fraction V)

- **2 g (100 ml)$^{-1}$** Ficoll 400

- **2 g (100 ml)$^{-1}$** Polyvinylpyrrolidone

- **Hybridisation buffer** (store at $-20^\circ$C)

  - **25 ml** Formamide
  - **10 ml** 20× SSC
  - **2.5 ml** 100× Denhardt’s solution
  - **10 ml** 50% dextran sulphate in distilled water
  - **500 µl** 10 mg ml$^{-1}$ herring sperm DNA

  Make up to 50 ml with MilliQ water

- **10× Tris-buffered saline (TBS)** (store at room temperature)

  - **23.6 g litre$^{-1}$** Tris base
  - **127 g litre$^{-1}$** Tris/HCl
Preparation of DIG-labelled probes

i) Perform PCR on purified AbHV DNA or a sample known to contain AbHV using a PCR DIG Probe Synthesis Kit (Roche Cat. No. 11 636 090 910) as per the manufacturers' instructions.
   a) Use AbHV.ORF66f1 (5’-TCC-CGG-ACA-CCA-GTA-AGA-AC-3’) AbHV.ORF66r2 (5’-CCC-GGA-CAC-CAG-TAA-GAA-C-3’) primer pair which amplifies an 848bp product from AbHV DNA.
   b) Use the following thermocycling profile: 95°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds. Complete the PCR with a final elongation at 72°C for 10 minutes.

Preparation of sections

i) Section paraffin-embedded tissue at 3 µM thickness and place onto Superfrost plus slides (Menzel Catalogue No. SF41296SP) and allow to dry.
ii) Heat sections at 65°C for 30 minutes and deparaffinise in two stages of xylene.
iii) Rehydrate by placing slides in absolute ethanol for 2 minutes followed by 90% ethanol for 2 minutes, 70% ethanol for 2 minutes and then into distilled water.
iv) Place slides in 0.2 N HCl for 20 minutes and rinse in distilled water for 5–10 minutes.
v) Apply 50–100 µl of 100 µg ml–1 proteinase K in Tris-buffered saline (TBS) and incubate at 37°C for 30 minutes.
vii) Rinse with 0.2% glycine for 2 minutes.
viii) Wash in running water for 10 minutes.
ix) Dehydrate sample in 70% ethanol for 2 minutes followed by 90% ethanol for 2 minutes and 100% ethanol for 2 minutes.
x) Allow slides to air-dry.

Hybridisation procedure

i) Make 100 µl hybridisation solution per tissue section (4× SSC, 5× Denhardt’s solution, 10 mg ml–1 herring sperm DNA, 10% dextran sulphate, 50% formamide, approximately 5 ng µl–1 probe).
ii) Heat the hybridisation solution to 95–100°C for 5 minutes to denature the probe and place on ice until ready for use.
iii) Apply sufficient hybridisation solution to cover the section (approximately 50 µl) and cover with a cover-slip.
iv) Heat the slides to 95°C for 5 minutes to denature the nucleic acid in the specimen. To heat the slides to 95°C a PCR heating block can be used or a purpose built hybridisation block such as the Invitrogen SPoT hybridiser.
v) Place the slides into a humidified chamber that has been preheated to 37°C and incubate at 37°C overnight (12–16 hours).

Post-hybridisation procedure

i) Remove cover-slips by immersing slides in 2× SSC at room temperature.
ii) Place slides in a rack and immerse in 2× SSC at room temperature. Use a rocker or shaker at slow speed to ensure complete washing of the slides.
iii) Wash, with gentle rocking/shaking, in 0.5× SSC (pre-warmed to 37°C) at 37°C for 15 minutes.
iv) Wash slides briefly in TBS buffer (Solution I) at room temperature.
v) Incubate slides in blocking solution (0.5% skim milk powder in TBS) for 30 minutes at room temperature.
vi) Cover sections with 100–200 µl of sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche Cat. No. 1093274) diluted 1 in 100 in blocking solution and incubate at room temperature for 1 hour.

vii) Wash in TBS buffer 3 × 3 minutes.

viii) Equilibrate in solution II (0.1 M Tris pH 8, 0.5 M NaCl, 0.1 M MgCl₂, pH 9) for 3 minutes at room temperature.

**Colour development**

i) Add 1 NBT/BCIP Ready-to-Use Tablet (Roche Cat. No. 11 697 471 001) to 10 ml of a 10% solution of polyvinyl alcohol (high molecular weight, 40–100 kD) in distilled water to prepare a ready-to-use staining solution.

ii) Cover the sections with the staining solution and place a cover-slip over them. Incubate in the dark for 3–4 hours in a humidified container, making sure that the slides do not dry out.

iii) Monitor the colour development by periodically checking the slides under a light microscope.

iv) If required the slides can be incubated, in the dark at room temperature, overnight.

v) Stop the reaction and remove the cover-slip by immersing the slides in distilled water.

vi) Wash the slides in running water for 5 minutes.

vii) Mount the slides with mounting medium (DAKO Cat. No. S3023) and a cover-slip.

**Interpretation of results**

Specific dark blue-black intra-cellular staining is indicative of the presence of viral DNA.

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 targeted surveillance and diagnosis of AVG are listed in Table 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 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. Methods for targeted surveillance and diagnosis**

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PLs = postlarvae; EM = electron microscopy; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

6. **Test(s) recommended for targeted surveillance to declare freedom from infection with abalone herpes-like virus**

The test recommended for targeted surveillance is qPCR on extracted nucleic acids from neural tissue of abalone.

7. **Corroborative diagnostic criteria**

7.1. **Definition of suspect case**

The presence of AbHV shall be suspected if at least one of the following criteria is met:

i) Presence of high mortality rates (up to 90%) associated with clinical signs of the disease as described in this chapter.

ii) Histopathology (ganglioneuritis) observed in neural tissue sections of a single abalone sample.

iii) Positive result by qPCR on at least one sample of abalone.

7.2. **Definition of confirmed case**

The presence of AbHV is considered to be confirmed if, in addition to the criteria in Section 7.1, one or more of the following criteria are met:

i) Positive result by qPCR on at least one repeat sample of abalone.

ii) Positive result by in situ hybridisation on neural tissue section.
iii) Positive result by conventional PCR and confirmation of AbHV sequence of the amplicon.

8. References


* * *

NB: There is an OIE Reference Laboratory for Infection with *Bonamia ostreae* (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).

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