Virus Research 165 (2012) 207–213

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Abalone viral ganglioneuritis: Establishment and use of an experimental immersion challenge system for the study of abalone herpes virus infections in Australian abalone

Serge Corbeil[a,*], Kenneth A. McColl[a], Lynette M. Williams[a], Ilhan Mohammad[b], Alexander D. Hyatt[a], Sandra G. Crameri[a], Mark Fegan[b], Mark St.J. Crane[a]

[a] CSIRO, Livestock Industries, Australian Animal Health Laboratory, 5 Portarlington Road, Geelong East, Victoria 3220, Australia
[b] Biosciences Research Division, Department of Primary Industries, 475 Mickleham Road, Attwood, Victoria 3049, Australia

ARTICLE INFO

Article history:
Received 19 November 2011
Received in revised form 14 February 2012
Accepted 16 February 2012
Available online 25 February 2012

Keywords:
Abalone viral ganglioneuritis
AbHV
Immersion challenge
Virus detection

ABSTRACT

In late 2005, acute mortalities occurred in abalone on farms located in Victoria, Australia. Disease was associated with infection by an abalone herpes virus (AbHV). Subsequently, starting in 2006, the disease (abalone viral ganglioneuritis; AVG) was discovered in wild abalone in Victorian open waters. Currently, it continues to spread, albeit at a slow rate, along the Victorian coast-line. Here, we report on experimental transmission trials that were carried out by immersion using water into which diseased abalone had shed infectious viral particles. At various time points following exposure, naïve abalone were assessed by an AbHV-specific real-time PCR and histological analyses including in situ hybridization (ISH). Results demonstrated that while exposed abalone began displaying clinical signs of the disease from 60 hours post exposure (hpe), they tested positive for the presence of viral DNA at 36 hpe. Of further interest, the AbHV DNA probe used in the ISH assay detected the virus as early as 48 hpe.

1. Introduction

In late 2005, an acute disease, abalone viral ganglioneuritis (AVG), associated with very high mortality (up to 100%) in farmed abalone, occurred on land-based farms in Victoria, Australia (Hooper et al., 2007). In May 2006, the disease was discovered in wild abalone, initially in the vicinity of the affected farms, and it subsequently spread along the Victorian coast-line. The initial mortalities on-farm involved blacklip abalone (*Haliotis rubra*), greenlip abalone (*Haliotis laevigata*) as well as hybrids of these two species. The precise extent of mortality in wild populations is difficult to determine partly due to the cryptic habitat of, in particular, young abalone; reported mortality in wild abalone at different locations has ranged between 10% and 90%. Following an initial investigation by State authorities, the clinical history, clinical signs of disease, and pathology were well-documented for this outbreak (Hooper et al., 2007), and, based on electron microscopic examination, an abalone herpes virus (AbHV) was identified as the putative aetiological agent (Tan et al., 2008). Currently, the disease continues to spread, albeit at a slow rate, along Victorian coastal waters, does not appear to be seasonal, and threatens abalone stocks throughout southern Australia. The disease appears to be restricted to abalone with no apparent effect on other molluscan or aquatic animal species. The lack of permanent molluscan cell lines (Chen and Wen, 1999) has hindered disease investigation and research on AbHV. Nevertheless, molecular tools have assisted not only in confirming that AbHV is the aetiological agent of AVG but also in the subsequent characterization of this virus (Corbeil et al., 2010; Savin et al., 2010). In our laboratory, infection trials were conducted using tissue homogenates prepared from moribund and dead wild abalone within Victorian waters. This allowed us to reproduce the disease experimentally and store infectious virus for extended periods of time. We have now developed an immersion challenge protocol that allows us to obtain information on the virus biology as well as its interaction with the host. This paper describes an immersion challenge system and, also, by means of a time-course study, the establishment of the earliest time-points from which molecular detection of the virus is achieved. In addition, the development of histological lesions in comparison with the onset of clinical signs of disease is reported.

2. Materials and methods

2.1. Experimental animals

Healthy abalone, blacklip (*H. rubra*) × greenlip (*H. laevigata*) hybrids (approximately 70 mm in diameter/2 years old), were obtained from a local abalone farm (Great Southern Waters Pty Ltd.,

* Corresponding author. Tel.: +61 3 5227 5254; fax: +61 3 5227 5555.
E-mail address: serge.corbeil@csiro.au (S. Corbeil).

0168-1702/$ – see front matter.
doi:10.1016/j.virusres.2012.02.017

Indented Head, Victoria) in a part of Victoria where there has been no history of AVG. In addition, abalone samples from this farm have consistently tested negative using the recently developed AbHV specific real-time (Taqman ORF-49) PCR test (Corbeil et al., 2010). The abalone were placed in tanks containing aerated, filtered, natural sea water maintained at 16–18 °C and fed small food pellets daily (Halo from Skretting Inc., Tasmania, Australia). All tanks underwent a 100% water change daily during each experiment. Abalone were harvested when they showed signs of morbidity (e.g., lethargy and difficulty to attach to the substrate).

All animal experiments were approved by the Australian Animal Health Laboratory Animal Ethics Committee in accordance with the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition.

2.2. Virus preparation, storage and titration

The Victorian isolate (Vic1) of AbHV was used in this study. Viral stocks were prepared from the pleuropedal ganglia and associated nerve cords of 16 moribund abalone from a previous experimental infection trial. In a class II safety cabinet, infected tissues were suspended in 12 mL sterile EMEM (Gibco) containing 10% foetal calf serum (Gibco) and homogenized on ice using a using a 10 cm^3 Dounce homogenizer for 4 min (yields approximately 0.5 g of homogenized tissue in a 15 mL volume). The homogenate was centrifuged at 1500 × *g* for 20 min at 4 °C. The supernatant containing the virus was removed, filtered (0.22 μM) and aliquots (250 μL) were frozen in liquid nitrogen. Storage in liquid nitrogen prolongs viral infectivity for longer time periods (at least 21 months) than storage at −20 or −80 °C (Corbeil et al., unpublished data). To investigate the potential presence of bacteria in the suspension, 50 μL of the prepared inoculum was plated on nutrient and horse blood agar and incubated at 15 and 20 °C for 48 h. No bacterial growth was observed on agar plates under these conditions. In addition no bacterial infection was observed by electron microscopic analysis and histopathological examination of tissues from infected abalone.

Four days prior to immersion challenge, 5 abalone were injected intra-muscularly with 0.1 mL of a 1:100 dilution of the prepared viral stock and maintained in 8 L of aerated sea water at 16–18 °C. Water was changed daily, and on day 4 post-injection the water from the tank was collected and used for the immersion challenges. A sample (140 μL) of the infectious water was taken for DNA extraction using the Viral RNA Qiagen kit (cat. # 52904) following the manufacturer's instructions. The relative viral titre was estimated by determining the copy number of the TaqMan PCR target present in the water. The target copy number was established using the AbHV specific real-time TaqMan PCR assay and a standard curve generated using a plasmid in which the PCR amplicon has been cloned (Corbeil et al., 2010). Titres were extrapolated to virus gene copy numbers per mL of water and per 20 mg of abalone tissue.

2.3. DNA extraction from abalone tissue

Nucleic acid from AbHV-infected and uninfected abalone tissues (approximately 20 mg of tissue including the target neural tissue surrounded by some muscle) was extracted using a QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions. Nucleic acid, bound to minicolumns, was eluted and resuspended in a final volume of 100 μL of AE buffer and quantified using a Nanodrop spectrophometer (ND-1000V.5.3.2, Nanodrop Technologies Inc.) (100 ± 20 ng μL^{-1}). DNA samples were not diluted to a specific concentration so as not to reduce the amount of viral DNA potentially present in samples. Samples were tested immediately after extraction or stored at −80 °C until tested.

2.4. Dose–response study

Four groups of eight or nine abalone were challenged by immersion for 20 h in 20 L of sea water (150 L size tanks) containing either 2.5×10^6, 2.5×10^5, 2.5×10^4 or 2.5×10^3 viral gene copies (v.g.c.)/mL. The negative control group consisted of 8 abalone immersed in sea water free of virus. All abalone were then transferred to individual tanks, each containing 2 L sea water at 16–18 °C that was changed daily. Animals were monitored daily for signs of morbidity. Based on reports from natural disease outbreaks and preliminary experimental infections, it was known that all moribund animals would die within 24 h of showing clinical signs of disease (e.g., loose attachment to the substrate). Therefore, moribund abalone were immediately sampled for examination by PCR (9 abalone) and histopathology (33 abalone). For sampling, abalone were dissected to expose the pleuropedal ganglion and nerve cords. The neural tissue was removed and placed in either: a tube on ice (for subsequent PCR) and transferred to −20 °C until DNA extraction; or, in 10% sea water formalin for a minimum of 24 h and then processed for histopathological/ISH analyses. Nucleic acid was extracted from frozen tissue samples using the QIAamp DNA® mini kit (Qiagen) following the manufacturer's instructions and processed for PCR analysis using real-time PCR (Corbeil et al., 2010).

2.5. Time-course study

Forty-eight naïve abalone were held in a tank containing 60 L of aerated sea water. At time 0 h, four abalone were euthanized and sampled as negative control tissues. The 44 remaining naïve abalone were then immersed in 18 L of infectious water containing $\sim 1.6 \times 10^6$ v.g.c./mL. After 6 h, the water volume was made up to 40 L using fresh sea water and, from then on, to maintain high water quality, 100% of the water was changed every 24 h (40 L) for the duration of the experiment. Four abalone were sampled at each of the following time points: 3, 6, 12, 24, 36, 48, 60, 72, 84, 96 and 108 hpe. They were processed for PCR (2 animals), and histopathology/ISH (2 animals). The AbHV TaqMan assay was used to establish C_T values and virus gene copy numbers per 20 mg of abalone tissue. Because preliminary work had shown that histological lesions were restricted to the neural tissues (Hooper et al., 2007; McColl et al., unpublished), histopathological examination in this study was restricted to the nervous system of experimental abalone.

2.6. Histology and in situ hybridization

The formalin-fixed tissue sections containing the pleuropedal ganglion and nerve cords were prepared by routine histological procedures including dehydration through an alcohol series, paraffin embedding, sectioning (3–6 μm), and staining with haematoxylin and eosin (for histopathological analysis) or treated with the ORF-66 AbHV probe (for ISH) (OIE, 2011).

2.7. Electron microscopy

Pleuropedal ganglion tissue was sampled from abalone at 72 hpe for examination by electron microscopy was fixed using 2.5% (v/v) glutaraldehyde in 0.1 M Sorensen's phosphate buffer and post-fixed in 1% (w/v) osmium tetroxide. The samples were washed in reverse osmosis water (3× 5 min), dehydrated in a graded series of ethanol (70% overnight 4 °C, 95% 20 min, 100% 2× 20 min), infiltrated in 100% Spurr's resin overnight, embedded and polymerized at 65 °C overnight. Ultrathin sections (90 nm) were cut and stained in uranyl acetate followed by lead citrate. Preparations were examined using a Hitachi H700 transmission electron microscope at 100 kV.