Australian abalone (*Haliotis laevigata*, *H. rubra* and *H. conicopora*) are susceptible to infection by multiple abalone herpesvirus genotypes

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ABSTRACT: From 2006 to 2012, acute mortalities occurred in farmed and wild abalone (*Haliotis* spp.) along the coast of Victoria, Australia. The disease (abalone viral ganglioneuritis; AVG) is associated with infection by an abalone herpesvirus (AbHV). The relative pathogenicity of 5 known variants of AbHV was evaluated on abalone stocks from different states in Australia. Results indicated that all virus variants (Vic1, Tas1, Tas2, Tas3 and Tas4) cause disease and mortality in all abalone stocks tested (greenlip, blacklip and brownlip). In order to avoid further AVG outbreaks in Australian wild abalone, strict regulations on the transfer of abalone stocks must be implemented.

KEY WORDS: Abalone viral ganglioneuritis · AVG · Abalone herpesvirus · AbHV · Experimental challenge · Susceptibility to AVG

INTRODUCTION

Abalone viral ganglioneuritis (AVG), an acute disease associated with very high mortality (up to 100%), was first observed in abalone *Haliotis laevigata* land-based farms in Victoria, Australia (Hooper et al. 2007). Histopathological and electron microscopic analyses of neural tissues (Tan et al. 2008), as well as subsequent genomic sequencing of purified virus (Savin et al. 2010) revealed the aetiological agent to be a herpesvirus, now designated abalone herpesvirus (AbHV). AVG is characterized by inflammation and necrosis of the nervous tissues of affected abalone (Hooper et al. 2007, Corbeil et al. 2012). Soon after its emergence on farms, AbHV was found in wild abalone, causing significant economic losses to the wild-capture industry in Victoria. This prompted the urgent development of molecular diagnostic assays for the detection of AbHV in order to help industry and regulatory authorities to manage the disease (Corbeil et al. 2010). In 2008, the virus was discovered in a processing plant in Tasmania,

and similar occurrences were observed annually from 2009 to 2012 (Ellard et al. 2012). Partial sequencing of all Tasmanian virus variants showed nucleotide variations in some genes (Cowley et al. 2012); however, it is not known whether these variations would have an effect on their pathogenicity. In addition, unlike Victoria, no disease outbreak was seen in Tasmanian wild *Haliotis* populations.

Abalone species such as *H. diversicolor* Reeve in China (Wang et al. 2004) and *H. diversicolor supertexta* (Chang et al. 2005) have also suffered herpesvirus infections, but no information is available on the diversity of viral strains involved. Similarly, in Australia, no study has been conducted to establish whether all Australian abalone species/stocks are susceptible to infection with AbHV, and whether AVG is the inevitable outcome of infection.

Crane et al. (2013) showed that surviving abalone from wild populations that had previously undergone AVG outbreaks had not developed resistance to the disease. It is not known whether all recognized variants of AbHV would have the same impact on Aus-

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tralian abalone stocks. Therefore, in order to better understand the epidemiology of AbHV and its potential effect on abalone stocks around Australia, laboratory challenge trials were performed using 5 genetically different variants of AbHV and the predominant abalone species from the temperate waters in the states of Tasmania, Victoria, South Australia (SA) and Western Australia (WA). Susceptibility was evaluated based on the presence or absence of virus DNA in abalone tissue and survival rate upon viral challenge.

MATERIALS AND METHODS

Origin and species of abalone

Healthy control abalone, blacklip *Haliotis rubra* × greenlip *H. laevigata* hybrids (approximately 70 mm in length and 2 yr old), were obtained from the Craig Mostyn Group, Jade Tiger abalone Pty Ltd, Indented Head, Victoria, where there has been no history of AVG. In addition, abalone samples from this farm have consistently tested negative using the AbHV-specific qPCR test (ORF-49 and ORF-66 qPCR; Corbeil et al. 2010, OIE 2015). These abalone were used as positive controls as well as to produce water containing infectious virus of each variant for the immersion challenge trials. The experimental abalone were blacklip, greenlip and brownlip *H. conicopora* obtained from the following suppliers: 888 Abalone Pty Ltd, WA (greenlip and brownlip; ~70 mm in length, ~3 yr old); Costal Seafarms Pty Ltd, Victoria (greenlip ~70 mm, ~3 yr; this stock of abalone originated from SA); Ocean Wave Seafoods Pty Ltd, Victoria (greenlip and blacklip; ~ 30 mm, ~2 yr and 65 mm, ~3 yr, respectively); and Abtas Marketing Pty Ltd, Tasmania (greenlip and blacklip; ~80 mm, ~4 yr old).

To allow the experimental abalone to acclimate to the laboratory, they were placed for 4 to 5 d in individual tanks containing aerated, filtered, natural seawater maintained at 16–18°C, and fed small food pellets daily (Halo; Skretting). During experiments, all tanks underwent a 100% water change every 2 d. Abalone were sampled for laboratory analysis when they showed signs of morbidity (e.g. lethargy and inability to attach to the substrate).

Preparation of infectious water

The Victorian variant Vic1 and the Tasmanian variants Tas1, 2, 3 and 4 of AbHV were used in this study.

Viral stocks were prepared according to the methodology described previously (Corbeil et al. 2012). Four days (Vic1, Tas1, 3 and 4) and 5 d (Tas2) prior to immersion challenge, 15 abalone were injected intramuscularly with 0.1 ml of a 1:100 dilution of the previously prepared viral stock and then maintained in 20 l of aerated seawater at 16–18°C. Water was changed daily, and on Days 4 or 5 post-injection (determined by the specific viral variant) the infectious water from the tank was collected and diluted in seawater at 1:2 for the immersion challenges.

Immersion challenge

Eight abalone per species were challenged by immersion in 1 l individual tanks of seawater for 20 h. All groups of abalone species in any given experiment were exposed to an identical viral challenge dose (1:2 dilution). The negative control group consisted of 8 abalone immersed in seawater free of virus in a separate room. After the 20 h exposure period, the challenge water was removed and fresh seawater (16–18°C) was added to each tank and changed every 2 d. Animals were monitored daily for signs of morbidity. Based on reports from natural disease outbreaks and previous experimental infections, it was expected that all moribund animals would die within 24 h of observing clinical signs of disease (e.g. loose attachment to the substrate). Therefore, any moribund abalone observed were immediately sampled for examination by PCR and histology. 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% seawater formalin for a minimum of 24 h and then processed for histopathological analysis. Nucleic acid was extracted from frozen tissue samples and detected as described below.

DNA extraction from abalone tissue

Nucleic acid from AbHV-challenged and -unchallenged control abalone tissues (approximately 100 mg of tissue including the target neural tissue surrounded by some muscle) was extracted (using Applied Biosystems Mag MAX Express 96 Viral Isolation Kit and the MagMAX Express-96 Magnetic Particle Processor) following the manufacturer's instructions, except that the final elution was made in 50 µl.

Viral DNA detection

Viral DNA was detected using qPCR (Corbeil et al. 2010) with the following modifications for the open reading frame (ORF)-66 qPCR (by homology to sequences of OsHV-1 in GenBank, ORF 66 is likely to encode a DNA primase small subunit): forward primer ORF66F1 (5′-TCC CGG ACA CCA GTA AGA AC-3′), reverse primer ORF66R1 (5′-CAA GGC TGC TAT GCG TAT GA-3′), and 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA)-labelled probe ORF66Prb1 (FAM-5′-TGG CCG TCG AGA TGT CCA TG-3′-TAMRA). The expected length of the amplicon is 146 bp. The thermal cycling conditions were 95°C for 59 s, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. C_T values <38 were considered positive for the presence of AbHV DNA.

Preparation of plasmid DNA positive control

In order to validate the results obtained with the experimental samples, the AbHV target amplicon (146 bp) was cloned into the pCR4 Blunt Topo vector (Invitrogen) according to the manufacturer's instructions. The recombinant plasmid from 1 clone was sequenced to confirm the presence of the amplicon. The plasmid, designated Topo-ORF66, was replicated in *E. coli* DH5α and extracted using a QIAprep kit (Qiagen) according to the manufacturer's instructions. The concentration of the plasmid Topo-ORF66 was determined in triplicate by nanodrop spectrophotometry using a wavelength of 260 nm and extinction coefficient of 50 ng cm ml^{-1}). The supercoiled plasmid Topo-ORF66 was heated at 95°C for 5 min and put on ice for 5 min to separate the plasmid strands which facilitates the qPCR amplification process.

Histology

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.

Statistical analysis

The log-rank (Mantel-Cox) test (GraphPad Prism® 5.02) was performed to compare the survival curves of abalone species challenged with the 5 AbHV strains.

RESULTS

Cumulative mortality

Morbidity and mortality occurred in all groups of abalone challenged with all AbHV variants during the trials (Fig. 1). No mortality occurred and no abnormal histology was observed among the negative control abalone.

Comparative susceptibility of 3 abalone species to AbHV variants

The log-rank (Mantel-Cox) test demonstrated no significant differences between abalone species challenged with AbHV variants within each challenge trial (results not shown) with the exception of Victorian blacklip abalone, which were more susceptible than the greenlip abalone upon challenge with AbHV Tas2 and Tas3 (Table 1).

Histopathology/qPCR analysis

All moribund abalone examined histologically demonstrated tissue lesions consistent with AVG (Hooper et al. 2007, Corbeil et al. 2012) (results not shown). No difference in histological lesions was observed between abalone species and virus variants.

All moribund abalone tested by the qPCR (Corbeil et al. 2010, OIE 2015) were positive for the presence of AbHV DNA. Amongst the abalone that survived the viral challenge, some were analysed by histology and tested by qPCR. In most cases, histological results were not definitive. However, 6 animals showed histological lesions typical of AVG, and amongst these, 4 tested positive by qPCR (Table 2). Overall, the mean C_T value of moribund abalone was much lower (higher viral load) than the mean C_T value of surviving abalone (Table 3).

DISCUSSION

The results show that greenlip, blacklip and brownlip abalone originating from the states of Victoria, Tasmania and SA, and the brownlip abalone from WA are susceptible to the 5 AbHV variants tested (Vic1, Tas1, Tas2, Tas3 and Tas4). Greenlip from WA are susceptible to AbHV Tas3 and Tas4 but could not be tested with the variants Vic1, Tas1 and Tas2 due to difficulty in maintaining enough greenlip abalone
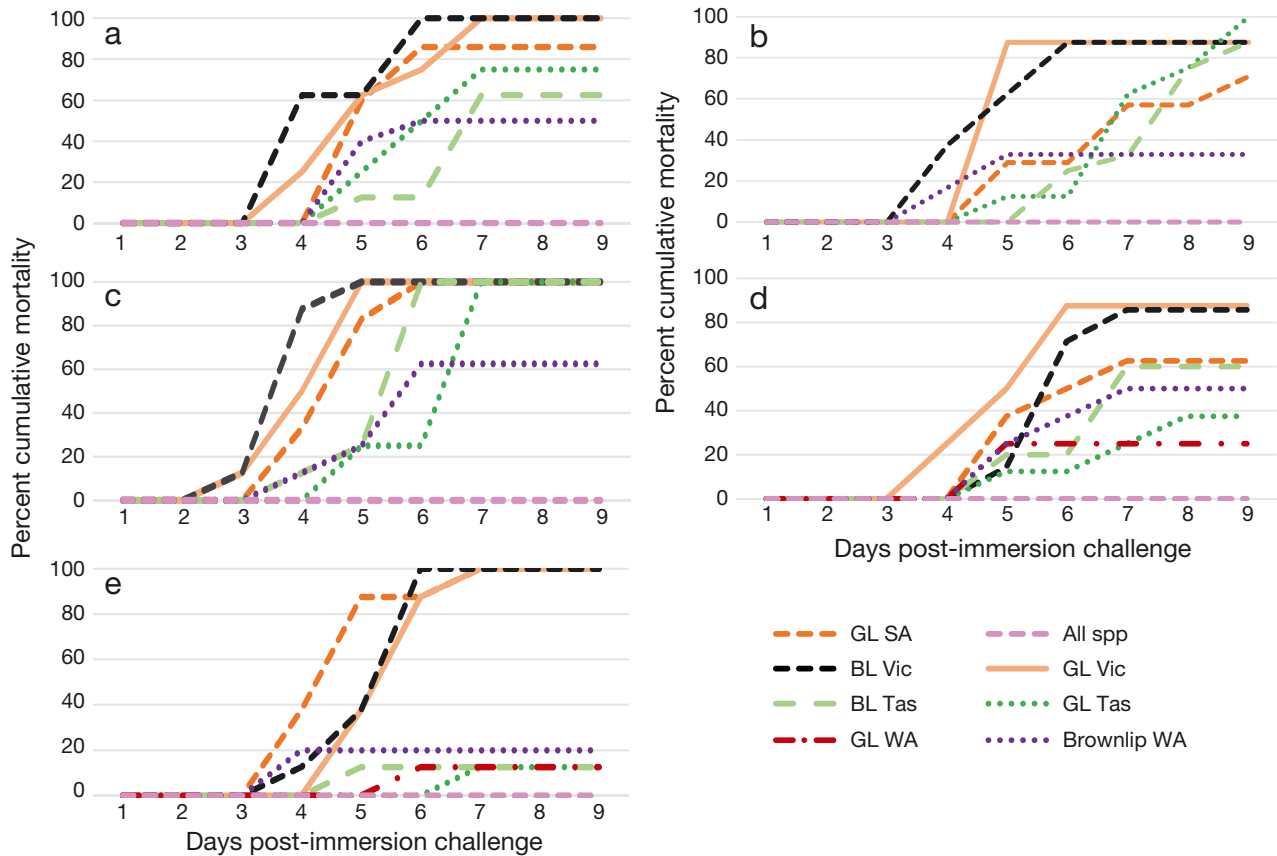
Fig. 1. Cumulative mortality curves for immersion trials of greenlip *Haliotis laevigata* (GL), blacklip *H. rubra* (BL) and brownlip *H. conicopora* abalone samples from populations originating from Victoria (Vic), Tasmania (Tas), South Australia (SA) and Western Australia (WA), following challenge with abalone herpesvirus (AbHV) genotypes (a) Vic1, (b) Tas1, (c) Tas2, (d) Tas3 and (e) Tas4. The difference between final cumulative mortality and 100% represents percentage survival. Note that curves from abalone originating from different states cannot be compared with each other

in healthy condition after transportation. Differences in pathogenicity between viral variants may exist; however, due to variation in viral growth and inability to titre infectious virus accurately, we could not establish differences in pathogenicity between the numerous challenge trials undertaken over a 2 yr period. Indeed, because AbHV has not been successfully grown in cell culture, and molecular titration measures viral DNA present in suspension rather

Table 1. Log-rank (Mantel-Cox) test on Victorian greenlip abalone *Haliotis laevigata* and blacklip abalone *H. rubra* challenged with abalone herpesvirus (AbHV) genotypes Tas2 and Tas3. Results indicate that blacklip abalone were more susceptible than greenlip abalone upon challenge with Tas2 and Tas3

AbHV	χ^2	df	p	Curves significantly different (p < 0.05)
Tas2	9	1	0.0027	Yes
Tas3	4.4	1	0.035	Yes

than viable viral particles, precise comparative pathogenicity is not yet achievable.

Our results show that all abalone species tested are susceptible to the AbHV variants, although statistical analyses indicated differences in mortality between Victorian blacklip abalone and the Victorian greenlip upon AbHV Tas2 and Tas3 challenges with the same infectious water. Although both species showed identical final percent mortality, morbidity occurred faster in blacklip upon challenge with the Tas2 variant and faster in greenlip upon challenge with the Tas3 variant. A slight discrepancy in weight may have caused this difference and is a phenomenon we have noticed in previous studies (authors' pers. obs.).

In most surviving abalone in these trials, C_T values in the tissues were much higher (lower virus load) than in the abalone that succumbed to the infection. This suggests that some abalone were infected later than others in the immersion challenge, an observation supported by the fact that some of the surviving abalone that were positive by qPCR for viral DNA

Table 2. Presence or absence of histological lesions and C_T values of experimental abalone *Haliotis* spp. surviving the virus challenge. A C_T value of ≥38 is considered negative for the presence of viral DNA. AbHV: abalone herpesvirus, WA: Western Australia

Sample	AbHV variant	Species	Location	Histological lesion	qPCR C_T value
1	Tas3	*H. laevigata*	Victoria	Absent	≥40
2	Tas4	*H. conicopora*	WA	Present	37.34
3	Tas1	*H. conicopora*	WA	Present	≥40
4	Tas3	*H. conicopora*	WA	Absent	≥40
5	Tas3	*H. conicopora*	WA	Absent	38.30
6	Tas3	*H. conicopora*	WA	Present	35.89
7	Vic1	*H. conicopora*	WA	Present	39
8	Vic1	*H. conicopora*	WA	Present	37.5
9	Vic1	*H. conicopora*	WA	Absent	39.5
10	Vic1	*H. conicopora*	WA	Present	≥40
11	Vic1	*H. conicopora*	WA	Absent	37.99

Table 3. Mean C_T values of experimental abalone *Haliotis* spp. surviving and succumbing to abalone herpesvirus (AbHV) challenge trials

Abalone sample	qPCR C_T value	
	Mean	SD
Sacrificed after trial	34.96	4.81
Harvested when morbid	16.35	7.74

also showed some histological lesions characteristic of AVG. The abalone may have become moribund within a few days had they not been sampled on Day 9 post-challenge. Further studies should determine whether some abalone can become sub-clinical carrier animals that could survive for an extended time (beyond the 9 d of this experiment).

Greenlip and brownlip abalone originating from WA were susceptible to AbHV Tas3 and Tas4 variants. In addition, the brownlip abalone were also susceptible to AbHV variants Vic1, Tas1 and Tas2.

Susceptibility of Tasmanian abalone to the viral variants originating from Tasmania raises a question. If Tasmanian variants are pathogenic in all abalone stocks including those from Tasmania, why has no outbreak of AVG been recorded in Tasmanian wild abalone populations (as has been seen in Victorian stocks)? Are the environmental conditions in the wild (stock density, marine currents, water temperature) so different from the experimental conditions that outbreaks did not occur? In that respect, Moore et al. (2000) demonstrated that for red abalone *Haliotis rufescens*, an increase in water temperature from 14.7 to 18.5°C enhances the severity of the pathogenic effects of rickettsia

'*Candidatus* Xenohaliotis californiensis,' the aetiological agent of withering syndrome. Similarly, Rogers-Bennett et al. (2010) showed that when red abalone infected by *X. californiensis* are exposed to warm water, they develop clinical signs of withering syndrome, and both males and females experience total reproductive failure. With regards to viral infection in molluscs, Pernet et al. (2015) showed that ostreid herpesvirus remains latent at low temperature but is reactivated and disease occurs in Pacific oysters when the water temperature increases to a threshold level.

In the case of AVG, perhaps the Tasmanian virus variants remain sub-clinical in Tasmanian wild abalone because environmental conditions (for example, water temperature) favour a latent or persistent infection. It would be interesting to hold a large number of wild Tasmanian abalone in the laboratory at slightly warmer temperatures than those recorded in Tasmanian waters, and look for evidence of reactivation of possible latent infections. A study by Corbeil et al. (2013) on heritability of resistance traits of greenlip abalone against AVG has shown that increased resistance to the disease varies according to the genotype of the species even without prior encounter with AbHV. Similarly, Pacific oysters are genetically diverse in terms of their susceptibility to ostreid herpesvirus infection (Dégremont 2013, Segarra et al. 2014, Dégremont et al. 2015). In addition, Friedman et al. (2014) showed that natural selection in black abalone can lead to natural recovery (from disease) of abalone populations over time under certain circumstances.

Regarding the biosecurity aspect, it remains crucial for Australian state government agencies to establish, and enforce, strict regulations that limit the transfer of potentially infected or carrier abalone stocks from one region to another in order to avoid further AVG outbreaks in Australian wild abalone.

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