Abalone herpes virus stability in sea water and susceptibility to chemical disinfectants

Serge Corbeil*, Lynette M. Williams, Jemma Bergfeld and Mark St.J. Crane
Australian Animal Health Laboratory, CSIRO Livestock Industries, Private
Bag 24, Geelong Victoria 3220 Australia

*Corresponding author at CSIRO, Livestock IndustriesAustralian Animal Health Laboratory 5
Portarlington Road, Geelong East, Victoria, 3220 Australia. ph:+61(3) 5227 5254 Fax:+61(3) 5227 5555
E-mail: serge.corbeil@csiro.au

ABSTRACT

Experimental immersion and injection challenge methodologies were developed to investigate
the effects of various physicochemical treatments on the abalone herpes virus (AbHV), an
emerging virus causing viral ganglioneuritis in abalone in Australia. To determine stability at
different temperatures, the virus was held at 4, 15, or 25°C for 1, 5, and 12 days prior to
immersion challenge of naïve abalone. Mortality curves indicated that when held for one day in
sea water at 4°C and 15°C the virus remained infectious and highly pathogenic. In addition, the
virus retained partial infectivity after 5 days held at 4°C. Histological examination of abalone
tissues following viral exposure confirmed the presence of lesions typical of abalone viral
ganglioneuritis in animals showing morbidity signs. An additional experiment was performed to
determine the virucidal efficacy of three disinfectants (calcium hypochlorite, Buffodine and the
non-ionic surfactant Impress). The disinfectants were used at various doses and timeframes to
treat AbHV prior to injection and immersion challenges. Results showed that Buffodine and the
non-ionic surfactant Impress were effective at inactivating the virus with no detectable adverse
effects on the abalone’s health. In addition, calcium hypochlorite showed a virucidal effect when
used on lower titers of virus prior to immersion challenge.

Key words: Abalone viral ganglioneuritis; herpes virus; pathogenicity; chemical treatments.
1. Introduction

In December 2005/January 2006, a disease outbreak caused high mortality rates in abalone from two land-based farms in Victoria, Australia. Two other, marine-based farms also experienced disease but to a lesser extent. Histopathology performed on moribund abalone indicated a ganglioneuritis – infiltration of haemocytes in multiple ganglia and nerves (Hooper et al., 2007). Examination by electron microscopy revealed the presence of a herpes-like virus (AbHV) in the pleuropedal ganglion (unpublished data). Subsequently, the virus and disease, abalone viral ganglioneuritis (AVG), spread to wild abalone in the vicinity of one of the affected farms and mortalities of wild populations of abalone continue to occur and spread along the Victorian coast.

Outbreaks of AVG have raised questions concerning biosecurity requirements and disinfection procedures to be implemented to limit the spread of the virus in farms, processing plants, as well as other coastal water facilities, equipment and premises where commercial and recreational fishing take place. Lack of knowledge on the biology of the virus and its ability to survive in sea water as well as its susceptibility to standard disinfection methods, makes it difficult to establish biosecurity measures aimed at virus inactivation to prevent the spread of infection. Furthermore, standard aquaculture disinfectants have not been specifically tested on AbHV to establish their virucidal efficacy. This study has investigated two aspects of AbHV biology. First the ability of the virus to remain infectious and pathogenic when held at three different temperatures 4, 15, and 25°C for three lengths of time, 1, 5, and 12 days prior to immersion challenge of naïve abalone. This mode of infection is aimed at reproducing under experimental conditions, virus spread and survival in the water column in the wild, in aquaculture facilities and processing plants. Information from these experiments will contribute to our knowledge on the epidemiology of
AbHV with regards to horizontal transmission and potential risks to infect abalone and cause disease. The second aspect of this study covers the virucidal efficacy of three chemical compounds (calcium hypochlorite, the iodophor Buffodine, and the non-ionic surfactant Impress) on AbHV and will inform industry and regulators on the conditions of use of these chemicals.

2. Materials and methods

2.1 Experimental animals

Healthy abalone, blacklip (*Haliotis rubra*) x greenlip (*H. laevigata*) hybrids (approximately 70mm in diameter), were obtained from a local abalone farm (Great Southern Waters Pty Ltd, Indented Head, Victoria) in an area of Victoria where there has been no history of abalone viral ganglioneuritis (AVG). In addition, abalone samples from this farm have consistently yielded negative results using the recently published AbHV specific real-time (Taqman ORF-49) PCR test (Corbeil et al., 2010). For all experiments the abalone were placed in aquaria containing aerated, natural seawater maintained at 16°C (representing water condition from local farms) (room temperature was maintained constant using central air-conditioning rather than cooling the water directly in each individual aquarium). During each experiment, animals were fed and each aquarium underwent a 100% water change daily. 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 Production of AbHV infectious water
Infectious water used for immersion challenge experiments was produced by injecting five naïve abalone intramuscularly in the foot with 100µL of the Victorian isolate of AbHV (designated Vic-1) (~1 x 10^5 viral gene copies/100µL) (c.f. titration method Corbeil et al., 2010). Inoculated animals were held in an aquarium containing 8L of aerated sea water with daily water change. Previous experiments had shown that water from day 4 post-inoculation contained high levels of infectious virus. After 4 days water was harvested, titrated using the AbHV TaqMan assay and used as challenge infectious water.

2.3 Stability in sea water

Infectious water prepared as described above was harvested and stored in 50 mL aliquots at 4°C, 15°C and 25°C for 12 days before immersion challenge day. The same procedure was performed twice more for producing infectious water to be held at 4°C, 15°C and 25°C for 5 days and 1 day prior to immersion challenge. A total of 9 aliquots of infectious water were held. On the day of challenge (day 0), the nine aliquots of infectious water were placed in nine different aquaria containing 8L of sea water (~12.5 x 10^4 viral gene copies/mL). Each 8L of infectious water were then distributed into 8 small aquaria (1L/tank). Eight naïve abalone were placed individually in these aerated aquaria and challenged for a period of 20 hours (Figure 1). A negative control group of abalone was immersed in sea water without virus. After the challenge, a daily water change was performed for the 10 day duration of the experiments.

2.4 AbHV susceptibility to chemical disinfectants: Injection challenge

Aliquots (1 mL) of Hank’s buffered salt solution (HBSS) containing AbHV Vic-1 isolate (~200 x 10^6 viral gene copies per mL) were placed into six tubes. Calcium hypochlorite (Sigma-Aldrich), Buffodine (Malaguna PTY LTD) and the non-ionic surfactant Impress (Ultimate
Cleaning Products NQ) were added to the tubes to obtain the final concentrations indicated in Table 1. A positive control virus preparation did not receive any chemical treatment. After 10 minutes held at 16°C the content of the six tubes were transferred to 6 Vivaspin columns - 30 kDa cut-off (Sartorius Stedim Australia PTY, LTD). The columns were then centrifuged for 10 minutes at 1200 x g (IEC Centra 7R bench top centrifuge) at 4°C. The filtrate was discarded and 2mL HBSS was added to each column. The process was repeated twice more. After the third centrifugation the virus was resuspended in 1 mL HBSS and kept on ice. Groups of 6 abalone were injected intramuscularly with 100 µL of treated virus (20 x 10^6 virus gene copies) using 1 mL syringe fitted with a 26G gauge needle (Terumo Inc). A positive control group received 100 µL virus suspension that had not been exposed to any chemical. Six abalone forming a negative uninfected control group received 100 µL of HBSS alone (no virus). Treatment control abalone received 100 µL of HBSS previously treated with calcium hypochlorite to evaluate its potential residual toxicity after filtration through the Vivaspin columns. Each abalone was placed in individual aquarium containing 1.5L of sea water and monitored daily for 10 days duration of the experiment. Animals that showed typical clinical signs of AVG were euthanised and samples were fixed in 10% buffered formalin in sea water for histological examination.

2.5 Calcium hypochlorite treatment of AbHV infectious water: Immersion challenge

Three liters of infectious water (1.67 x 10^6 viral gene copies/mL) were distributed into three different aquaria (1L per tank) and were either left as is (positive control water) or treated with calcium hypochlorite to a final concentration of 10 and 15ppm. Two more aquaria contained normal sea water (virus-free) were also treated with calcium hypochlorite to a final concentration of 10 and 15ppm to evaluate any toxicity effect on abalone during and after immersion.
challenge. Waters were held at 16°C for 15 minutes and then water samples were taken to measure the level of residual free chlorine (ExStik CL200 Chlorine meter, Envco – Environmental Equipment). Following this treatment, 100mL of each aquarium/group were added (diluted) to 900mL of sea water in five new aquaria. Healthy abalone were added to the new aquaria (6 abalone per aquarium containing the virus and 2 abalone per aquarium containing control water now containing diluted calcium hypochlorite). Six abalone were also added to an aquarium containing sea water only and used as negative uninfected control group. Abalone were challenged for only 40 minutes to limit their contact with residual calcium hypochlorite, before transfer to individual aquaria containing 1.5L of fresh sea water. Water was changed daily for the duration of the experiments.

2.6 Histopathology scoring

The formalin-fixed tissue sections containing the pleuropedal ganglion, nerve cords and peripheral nerves were prepared by routine histological procedures including dehydration through an alcohol series, paraffin embedding, sectioning (3-6 µm), and staining with haematoxylin and eosin. Typical AVG lesions have been previously described and shown by Hooper et al. (2007), however, for this study lesions in tissues were evaluated according to the following scoring system:

Perineural sheath (PNS) 0: No viral lesion, 1: Scattered mild oedema and/or increased cellularity, 2: Extensive disruption of PNS by oedema and/or increased cellularity, 3: PNS almost completely disrupted by oedema and/or increased cellularity.

Grey matter (GM) lesions 0: No viral lesion L, 1: occasional neuronal necrosis; presence of small numbers of haemocytes and/or glia, 2: moderate numbers of neurons remaining, but
haemocytes/glia form the majority of cells in the GM: no (or almost no) recognizable neurons in the GM.

White matter (WM) lesions 0: No viral lesion, 1: a slight increase in cellularity, 2: a moderate increase in cellularity, 3: a heavy increase in cellularity.

Transverse commissure lesions 0: No viral lesion, 1: a slight increase in cellularity of commissures, 2: a moderate increase in cellularity, 3: a heavy increase in cellularity.

Peripheral nerve lesions 0: No viral lesion, 1: a slight increase in cellularity in occasional, or many, nerves, 2: a moderate increase in cellularity in occasional, or many, nerves, 3: a severe increase in cellularity in occasional, or many, nerves.

2.7 Statistical analysis

Survival curves log-rank (Mantel-Cox) test was performed on the data (Graph Pad Prism version 5.02).

3. Results

3.1 AbHV stability in sea water

Groups of abalone undergoing immersion challenges with AbHV held at 4°C for periods of 1 day and 5 days showed cumulative mortalities of 85 and 12.5% respectively (Fig 2a). Statistical analyses indicated that the 85% mortality curve was significantly different from the negative control group (P = 0.0001) but the 12.5% mortality was not different from the negative control group (P = 0.2). No mortality was recorded in the group exposed to the virus held for 12 days at 4°C prior to challenge (Fig 2a). Virus held at 15°C for 1 day induced 75% mortality in abalone (P
However, virus held for 5 and 12 days did not cause any mortality (Fig 2b). Amongst the groups of abalone challenged with virus held at 25°C only 12.5% of the abalone died in the 1 day group (Fig 2c) but the difference in mortality curve was not statistically significant to the negative control group (P = 0.2).

Challenged abalone that showed morbidity and clinical signs of AVG were harvested and examined for histopathology in their pleuropedal ganglion, nerve cords, and peripheral nerves. AVG lesions were observed in tissues of abalone challenged with the virus held at 4°C for 1 day (Tab 2). The one animal that died after being challenged with the virus held 4°C for 5 days showed tissue damage too advanced for proper histological evaluation. Abalone challenged with the virus held at 15°C for 1 day also showed AVG lesions in their tissues (Tab 2) (some animals that succumbed to this challenge had degraded tissues and could not be analysed). The abalone that died after challenge with the virus held at 25°C for 1 day did not show lesion in its tissues (results not shown). All other challenged abalone survived for the duration of the experiment and did not show signs of disease. Histological examination did not reveal any evidence of viral infection in these animals (results not shown). The negative control abalone survived for the duration of the experiment and no lesions typical of AVG were observed.

3.2 AbHV susceptibility to chemical disinfectants

Abalone injected with AbHV alone showed a typical mortality curve reaching 100% within 7 days post-challenge (Fig 3). The group of abalone injected with the virus treated with 5 and 10ppm of calcium hypochlorite showed mortality rates of 100% and 66% respectively (Fig 3). In addition, the 66% mortality rate of the 10ppm group of abalone was delayed by two days in comparison to the 5ppm group. Statistical analysis showed a significant difference between these
mortality curves and the negative control group curve ($P = 0.05$). Histological examination of these moribund animals revealed typical AVG lesions in their neural tissues (Table 3). The HBSS injected animals as well as all other groups of abalone challenged with treated virus (Buffodine and Impress) remained healthy for the duration of the experiment (Fig 3). Histological examination of the target tissues for these animals revealed normal appearance with no AVG lesions (results not shown).

In the injection experiment, groups of abalone injected with the virus treated with 5 and 15ppm of calcium hypochlorite showed mortality rates of 100% ($P = 0.03$). Again, the mortality curve of the 15ppm group showed a delay of two days compared to the 5ppm group. The virus treated with 20ppm of calcium hypochlorite induced 66% cumulative mortality (Fig 4), however, there was no difference with the accumulated 100% mortality of the positive control group (untreated virus) ($P$ value 0.44). Abalone injected with filtered HBSS solutions (without virus) containing calcium hypochlorite (to determine effect of chemical residue on abalone) all survived and did not show any lesions in their tissues (results not shown).

In the immersion challenge experiment, the group of abalone exposed to untreated infectious water showed a 50% cumulative mortality (Fig 5). In this group, three abalone out of six showed histopathology typical of AVG (Table 4). Groups of abalone exposed to infectious water treated with 10 and 15 ppm calcium hypochlorite (1.5 and 2 ppm of residual chlorine respectively after 15 min exposure) all survived the challenge and did not show histopathological legions in their tissues (results not shown). Abalone exposed to the calcium hypochlorite treated water only (no virus) all survived (Fig 5) and did not show any legions in their tissues (results not shown).
4. Discussion

Knowledge of the ability of an aquatic virus to spread through water and infect its host is of prime importance for its control and/or eradication. The development of an experimental infection model for AbHV in abalone in this experiment allowed us to conclude that in “ideal conditions” (pristine seawater with no other biological or physical factors) AbHV can survive in the water column at 15°C, remain infectious and cause disease 1 day after being shed in the water. It is likely that it could do so for up to 2 to 3 days although its infectivity/pathogenicity would be reduced in comparison to 1 day post-shedding (Fig 2b 85% mortality rate). Results also showed that increase in water temperature is not favourable for the virus survival (Fig 2c).

The results also confirm that direct contact between abalone is not required for horizontal transmission of this virus, as demonstrated in a previous study involving the Taiwanese isolate of an abalone herpes-like virus (Chang et al., 2005). Moreover, the virus appears to be stable for at least 1 day and perhaps even longer in seawater at 15°C, under experimental conditions. More natural conditions, i.e. the presence of other biological and physical factors, may modulate virus survival and water currents may influence the rate of spread. These results also show that virus survival is reduced at higher temperatures suggesting that transmission is more likely in the winter than in summer.

It is well recognised that for any infectious disease, prevention is the most effective control strategy and that use of chemical disinfectants plays an important part of this strategy (OIE 2010). Chemical disinfectants are commonly used in aquaculture settings to reduce or prevent transmission of pathogens on eggs as well as for the treatment of hatchery influent or effluent waters, contaminated raceways, equipment and other fomites. Chlorine and iodophors have been
found effective virucidal agents against fish pathogens such as infectious pancreatic necrosis virus (Elliott and Amend, 1978), infectious salmon anaemia (Smail et al. 2004), striped jack nervous necrosis virus (Arimoto et al., 1996), and white spot syndrome virus (Balasubramanian et al., 2006). In some cases the disinfectant efficacy depended on the concentration of the virus, the water pH and mostly the presence of organic matter in the water. Results presented here on virucidal efficacy of disinfectants also showed that iodophors such as Buffodine and the non-ionic surfactant Impress are very effective at inactivating AbHV. At relatively high viral titres Buffodine and Impress treated virus suspensions precluded any mortality in abalone challenged by intramuscular injection (Fig 3).

It has been suggested that using high viral titres in virucidal tests has certain drawbacks including possible aggregation of viral particles making them more resistant to treatments (Thurman and Gerba, 1988; Papageorgiou et al., 2001). This may explain why calcium hypochlorite had only limited virucidal activity against AbHV (Fig 3 and 4, injection experiments). Therefore, extended exposure time to chlorine would be needed in facilities where high organic matter content is present in the water.

Further criticism was raised regarding disinfection experimentations that used high concentrations of virus; the use of such high concentrations of virus is unlikely to represent the real-world situation in hatcheries (Papageorgiou et al., 2001). It has been suggested that the virus would more likely be dispersed in the flow-through waters at low concentration (Smail et al., 2004). In order to reproduce more realistic aquaculture facility conditions, infectious water containing lower AbHV titers (1.67 X 10^6 virus gene copies/mL) was treated with calcium hypochlorite followed by abalone challenge by immersion. Results indicated that 1.5 and 2.0
ppm of residual chlorine were sufficient to inactivate AbHV during a 15 minutes exposure. Some disinfectants may be more efficacious at a temperature of 20°C, however, the testing carried out at 16°C reflected the lower ambient temperatures for abalone facilities.

Histological observations of tissues from challenged abalone showed that abalone injected with AbHV or with AbHV treated with calcium hypochlorite had increased cellular infiltration and/or lesions typical of AVG (Hooper et al., 2007) (Table 2). However, animals immersed in infectious water treated with calcium hypochlorite did not show signs of disease or histopathology, suggesting that in aquaculture facilities virus loads could be inactivated using calcium hypochlorite, Buffodine or a non-ionic surfactant within a relatively short exposure time.

5. Conclusion

To our knowledge this is the first report on the establishment of an experimental infection model for the testing of physical and chemical factors on a moluscan virus. Results indicated that AbHV stability in the water column is modulated by temperature and loses its infectivity/pathogenicity within a few days under experimental conditions. The non-ionic surfactant Impress and the iodophor Buffodine are very effective virucidal agents while calcium hypochlorite is also effective but on lower virus concentrations. The non-ionic surfactant Impress is commercially available, is non-toxic and relatively inexpensive and would appear to be a disinfectant of choice for use in the treatment of abalone farming equipment (e.g. raceways, fomites, boots etc) as well as for professional abalone divers (e.g. boats, diving gear etc.).

Acknowledgements
The authors are grateful to the staff of Great Southern Waters Pty Ltd, Indented Head, Victoria for providing experimental abalone and seawater. We also thank Jean Payne and Jenni Harper from the histology laboratory at AAHL for assistance with processing abalone samples and providing paraffin blocks and H & E stained slides. This work was undertaken as part of FRDC Project No. 2009/032 (Aquatic Animal Health Subprogram: Characterisation of abalone herpes-like virus infections in abalone), and was supported by funding from the FRDC on behalf of the Australian Government.

References


