Aquaculture 326-329 (2012) 20–26

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

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

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ARTICLE INFO

Article history:
Received 6 September 2011
Received in revised form 8 November 2011
Accepted 9 November 2011
Available online 26 November 2011

Keywords:
Abalone viral ganglioneuritis
Herpes virus
Pathogenicity
Chemical treatments

ABSTRACT

Experimental infection models using immersion and injection challenges 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 1 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 durations 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 titres of virus prior to immersion challenge.

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 Examination by electron microscopy revealed the presence of a herpes-like virus (AbHV) in the pleuropedal ganglion (unpublished data).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), was observed in wild abalone in the vicinity of one of the affected farms and, currently, mortalities of wild populations of abalone continue to occur and spread along the Victorian coast approximately from Blue Nose to the west up to Cape Otway to the east. Affected reefs have suffered up to 95% mortality of their abalone populations (Victorian Abalone Diver Association, 2011). Most die-offs occurred in winter suggesting that colder water temperatures favour virus spread. To date, the abalone species *Haliotis rubra*, *Haliotis laevigata* and the hybrid of these species have shown susceptibility to AVG. In addition, juvenile and mature abalone are equally susceptible (unpublished results).

To limit the spread of the virus following outbreaks of AVG issues have been raised concerning biosecurity requirements and disinfection procedures to be implemented 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 periods of time, 1, 5, and 12 days prior to immersion challenge of naïve abalone. This mode of infection is aimed at simulating, under experimental conditions, virus spread and survival in the water column in the wild, in aquaculture facilities and in 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.

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0044-8486/$ – see front matter.
doi:10.1016/j.aquaculture.2011.11.031

2. Materials and methods

2.1. Experimental animals

Healthy abalone, blacklip (*H. rubra*) x greenlip (*H. laevigata*) hybrids (approximately 70 mm in diameter/2 years old), 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, filtered natural sea water maintained at 16 °C (representing water conditions in local farms) using central air-conditioning to maintain a constant room temperature, rather than cooling the water directly in individual aquarium. During each experiment, animals were fed commercial pellets (Halo from Skretting Inc., Tasmania, Australia) and each aquarium underwent 100% water changes 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

Virus stock was previously obtained from AbHV infected abalone tissues, homogenised, in Eagle's Minimal Essential Medium containing 20% foetal bovine serum, with a mortar and pestle on ice, filtered and stored in liquid nitrogen, until use. Infectious water used for immersion challenge experiments was produced by injecting five naïve abalone intramuscularly in the foot with 100 µL stock virus of the Victorian isolate of AbHV (designated Vic-1) (~1×10^5 viral gene copies (v.g.c.)/100 µL) (c.f. titration method in Corbeil et al., 2010). Inoculated animals were held in an aquarium containing 8 L aerated sea water with daily 100% water changes. Previous experiments had shown that water from day 4 post-inoculation contained high levels of infectious virus. After 4 days, the water was harvested, titrated using the AbHV TaqMan assay and used to generate 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. 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 8 L of sea water (providing a final viral titre of ~12.5×10^4 v.g.c./mL which ensures that 100% mortality will result after immersion challenge with control untreated virus) Each 8 L of infectious water was then distributed into 8 small aquaria (1 L/tank). Eight naïve abalone were placed individually in these aerated aquaria and challenged for a period of 20 h (Fig. 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 (~200×10^6 v.g.c./mL) were placed into 6 tubes (2 mL volume). Calcium hypochlorite (Sigma-Aldrich), Buffodine (Malaguna PTY LTD) or the non-ionic surfactant *Impress* (Ultimate Cleaning Products NQ) was added to the tubes to obtain the final concentrations specified in Table 1. A positive control virus preparation did not receive any chemical treatment. After 10 min incubation at 16 °C the contents of the 6 tubes were transferred to 6 Vivaspin columns—30 kDa cut-off (Sartorius Stedim Australia PTY, LTD). The columns were then centrifuged for 10 min at 1200×*g* (IEC Centra 7R bench top centrifuge) at 4 °C. The filtrate was discarded and 2 mL 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 (20×10^6 v.g.c.) using a 1 mL syringe fitted with a 26 G gauge needle (Terumo Inc). This virus titre is approximately 100,000 times higher than that required to kill an abalone by injection. Abalone in the 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 aquaria containing 1.5 L of sea water and monitored daily for the 10 days duration of the experiment. Animals that showed typical morbidity/clinical signs of AVG (e.g. lethargy and difficulty to attach to the

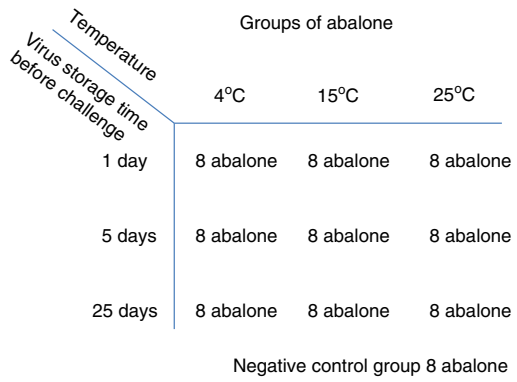

Fig. 1. Diagram showing various treatments for immersion challenge of abalone.

Table 1
Summary of the experimental groups to determine efficacy of chemical disinfectants.

Injection trial 1		
Chemical treatment	Low concentration	Medium concentration
AbHV + non-ionic surfactant *Impress*	1% (6 abalone)	5% (6 abalone)
AbHV + *Buffodine*	50 ppm (6 abalone)	nd
AbHV + calcium hypochlorite	5 ppm (6 abalone)	10 ppm (6 abalone)
Calcium hypochlorite alone	5 ppm (2 abalone)	10 ppm (2 abalone)
The positive control group (6 abalone) received AbHV in HBSS. The negative control group (6 abalone) received HBSS only. nd: Not done.		

Injection trial 2			
Chemical treatment	Low concentration	Medium concentration	High concentration
AbHV + calcium hypochlorite	5 ppm (6 abalone)	15 ppm (6 abalone)	20 ppm (6 abalone)
Calcium hypochlorite alone	5 ppm (2 abalone)	15 ppm (2 abalone)	20 ppm (2 abalone)

The positive control group (6 abalone) received AbHV in HBSS.
The negative control group (6 abalone) received HBSS only.