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Efficiency of Chlorine as a Disinfectant Against Monodon Baculovirus (MBV)

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Abstract

The effect of chlorine in the form of calcium hypochlorite solution on the infec-tivity of Monodon Baculovirus (MBV) for *Penaeus monodon* postlarvae in a bioassay was investigated using several concentrations and exposure times. MBV was inacti-vated by a 24-hour exposure to 1,000 $mg \cdot l^{-1}$ chlorine. Lower concentrations for more time or higher concentrations for less time reduced the prevalence of infections but did not eliminate them. The presence of small amounts of host tissue did not reduce the effectiveness of the chlorine.

Introduction

Monodon Baculovirus, MBV, was first described by Lightner and Redman (1981) in adult, laboratory-reared *Penaeus monodon*. The virus has since been found in a number of penaeid species throughout the Indo-Pacific region and in other areas where imported broodstock have been used (Paynter 1989). Shrimp become infected by ingesting virions released by carrier broodstock or by infected siblings (Paynter et al. 1992). Contaminated water, equipment and personnel allow transmission of viruses (Amend and Conte 1982).

Chlorine is commonly used as a disinfectant in culture facilities of finfish and specific disinfection recommendations exist for a number of fish diseases (Desautels and MacKelvie 1975; Amend and Conte 1982). A common source, calcium hypochlorite, is exempt from registration by the United States Food and Drug Administra-

tion as a disinfectant for tanks, raceways and utensils used in aquaculture (Schnick 1988). LeBlanc and Overstreet (1991) found that *Baculovirus penaei* (BP) was inactivated by chlorine concentrations of 200 $mg \cdot l^{-1}$ when treated for 1 hour and 1,600 $mg \cdot l^{-1}$ when treated for 20 seconds. In Australian shrimp hatcheries, MBV is controlled by destroying infected stock and disinfecting tanks, surfaces and equipment with a range of disinfectants (Paynter and Lester 1991), particularly chlorine as it is relatively cheap, accessible and safe. Here we report the susceptibility of MBV to chlorine disinfection.

Materials and Methods

Penaeus monodon postlarvae (PL 5-6) were collected from a hatchery in southeast Queensland, Australia, and determined to be MBV-free by histological examination. The hatchery did not have a history of MBV infection. The postlarvae were kept at 28°C in 33 ppt seawater at a density of $100 \cdot l^{-1}$ postlarvae. They were fed live brine shrimp nauplii *(Artemia salina)*. Water was changed daily and 4 ppm (as supplied) Furazolidone (Sigma, F9505, St. Louis, Missouri) added to each container for the first 8 days of each experiment.

A homogenate of virus infected tissue was made from *P. monodon* shrimp (PL 8-9) stored at -70°C, which had been obtained from a batch of postlarvae known to have MBV infection as confirmed by J. Paynter in 1991. For each of the 11 trials, 3 g of infected shrimp was thawed and added to 5 ml of 0.15M phosphate buffered saline, pH 7.2, containing 1mM disodium EDTA (Opdebeeck et al. 1988) and homogenized.

The homogenate was exposed to either 50, 200, 1,000 or 2,000 $mg \cdot l^{-1}$ of total available chlorine, produced by adding calcium hypochlorite (Olin HTH Granular Chlorine, approximately 65% active) to seawater.

Two vials were filled with 5 ml chlorinated seawater at each concentration and 2 ml viral homogenate was added to each vial. Vials were stored in the dark at 4°C to slow the action of digestive enzymes and lysozymes released during homogenization. After different time periods (Table 1) one vial from each pair was added to the water of a container of MBV-free *P. monodon* postlarvae (PL5-

Table 1. Initial chlorine (IC) concentration and exposure times used to treat viral homogenates before postlarvae were infected. Final Chlorine (FC) concentrations were measured after the homogenates were exposed.

Trial	Exposure time	IC ($mg \cdot l^{-1}$)	FC ($mg \cdot l^{-1}$)
1	1 hour	50	25
2	5 minutes	200	175
3	1 hour	200	150
4	8 hours	200	100
5	24 hours	200	100
6	30 seconds	1,000	1,000
7	5 minutes	1,000	800
8	24 hours	1,000	375
9	30 seconds	1,600	1,600
10	60 seconds	1,600	1,000
11	8 hours	1,600	500

6). Shrimp were exposed to infected tissue for 4 hours, after which the water was changed, the shrimp fed and 4 ppm Furazolidone (Sigma, F9505, St. Louis, Missouri) added. The second vial of each pair was used to measure chlorine concentration before the homogenate was added (initial concentration) and after exposure (final concentration) using a "Palintest" comparater and reagent tablets. Each treatment was conducted in duplicate.

Uninfected shrimp were kept as negative controls and shrimp exposed to 2 ml homogenate, which had not been treated with chlorine, for 4 hours were kept as positive controls.

Samples of 12 shrimp were removed from each container on days 6, 8, 10 and 12 of each trial and fixed in Davidson's fixative for histology (Humason 1968). Sections were stained with hematoxylin and eosin. Each shrimp was given an infection rating of between 0 and 4, where 0 indicated that no MBV occlusion bodies (OBs) were present, 1 that up to 10% of cells contained OBs, 2 that 10-40% of cells contained OBs, 3 that 40-70% of cells contained OBs, and 4 that 70-100% of cells contained OBs (Paynter et al. 1992). Mean abundance of OBs for each sample was calculated from the sum of infection ratings divided by the sample size.

To evaluate the degree of protection of virions within clumps of host tissue, a homogenate of 15 g infected tissue in 28 ml buffered saline was prepared and centrifuged at 5,000 G (1,800 rpm) for 10 minutes using a "Beckman" Model TJ-6 tabletop centrifuge. The

supernatant and pellet were separated and the pellet resuspended to the same volume as the supernatant. Supernatant and suspension were exposed separately to three calcium hypochlorite treatments; 50 $mg \cdot l^{-1}$ for 1 hour, 200 $mg \cdot l^{-1}$ for 1 hour, and 1,600 $mg \cdot l^{-1}$ for 60 seconds. Ten ml of chlorinated seawater was added to vials containing either 4 ml supernatant or 4 ml pellet.

A nonparametric Mann-Whitney U-test (SAS Institute Inc., North Carolina, USA) was used to analyze differences in OB abundance between groups of infected shrimp.

Results

Chlorine solution at 1,000 $mg \cdot l^{-1}$ completely inactivated the virus after 24 hours exposure (Fig. 1). Three of the 11 treatments investigated significantly reduced MBV OB abundance for the entire experimental time, but did not inactivate the virus: 200 $mg \cdot l^{-1}$ for 8 hours and 24 hours, and 1,600 $mg \cdot l^{-1}$ for 8 hours. Chlorine treatments of 50 $mg \cdot l^{-1}$ for 1 hour, 200 $mg \cdot l^{-1}$ for 1 hour and 5 minutes, 1,000 $mg \cdot l^{-1}$ for 30 seconds and 5 minutes and 1,600 $mg \cdot l^{-1}$ for 30 seconds did not significantly reduce the abundance of MBV OBs (Table 2).

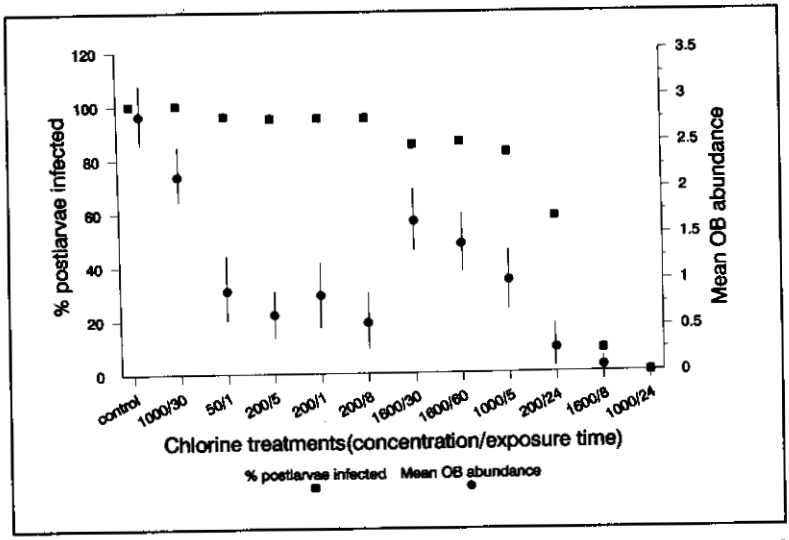

Fig. 1. Mean abundance of MBV occlusion bodies (OB) and the percentage of postlarvae infected 12 days after exposure to viral homogenates which had been treated with chlorine solutions of different concentrations for different exposure times. Vertical bars = 2x standard error either side of the mean.

Table 2. The percentage of postlarvae infected, the mean abundance of MBV occlusion bodies (OBs) and the efficiency of chlorine in reducing MBV infection, on each sampling day for each chlorine treatment.

Chlorine treatment	Day 6 % inf.	Day 6 OB abund.	Day 6 Sig. red. inf.	Day 8 % inf.	Day 8 OB abund.	Day 8 Sig. red. inf.
50/1	83%	0.83	-	59%	0.50	-
200/5	80%	0.87	-	59%	0.64	-
200/1	80%	0.83	-	76%	0.85	-
200/8	40%	0.40	x	26%	0.26	x
200/24	21%	0.42	x	21%	0.42	x
1000/30	70%	1.38	-	95%	1.27	-
1000/5	81%	1.00	-	96%	2.10	-
1000/24	0	0	*	0	0	*
1600/30	75%	0.75	-	83%	1.16	-
1600/60	86%	1.01	-	90%	1.00	-
1600/8	8%	0.08	x	8%	0.08	x
none (control)	100%	1.65		100%	2.10	

Chlorine treatment	Day 10 % inf.	Day 10 OB abund.	Day 10 Sig. red. inf.	Day 12 % inf.	Day 12 OB abund.	Day 12 Sig. red. inf.
50/1	90%	1.93	-	94%	0.82	-
200/5	91%	2.06	-	92%	0.62	-
200/1	95%	1.90	-	96%	0.83	-
200/8	81%	1.16	x	94%	0.55	x
200/24	43%	0.69	x	54%	0.24	x
1000/30	100%	2.03	-	100%	2.15	-
1000/5	90%	1.59	-	82%	1.00	-
1000/24	0	0	*	0	0	*
1600/30	91%	1.57	-	85%	1.65	-
1600/60	21%	1.18	x	86%	1.43	-
1600/8	5%	0.10	x	9%	0.10	x
none (control)	100%	2.42		100%	2.87	

x = treatment significantly reduced infection
- = treatment did not significantly reduce infection
* = MBV Inactivated

Total chlorine concentrations for all treatments decreased during exposure to the virus (Table 1).

MBV OBs were never seen in the hepatopancreocytes of the uninfected control postlarvae.

MBV OBs and free virions within the supernatant of the clarified tissue were not inactivated by any of the chlorine treatments. OB abundance levels for suspension and supernatant were not statistically different for any of the chlorine treatments (Table 3).

Table 3. Mean abundance of MBV occlusion bodies (OBs) and the percentage of postlarvae infected, 12 days after infection with either the supernatant or suspension of viral homogenate that had been treated with chlorine.

Chlorine treatment	Supernantant		Suspension	
	% infected	OB abundance	% infected	OB abundance
50 $mg \cdot l^{-1}$, 1 hour	92	2.39	100	2.42
200 $mg \cdot l^{-1}$, 1 hour	100	2.67	96	2.30
1,600 $mg \cdot l^{-1}$, 60 seconds	100	2.26	100	2.64
none (control)	100	2.77	100	2.21

Discussion

Oral ingestation of feces from infected shrimp is the main source of MBV infection (Chen et al. 1992; Paynter et al. 1992). Eradication of MBV within the hatchery is possible by ensuring that all surfaces and equipment which come into contact with the postlarvae are free of MBV OBs.

Wyban and Sweeney (1991) recommended that hatchery tanks be treated with a 1,600 ppm solution of chlorine and left for several hours. In our work, MBV was not inactivated by this treatment. Treating equipment, plumbing and floor surfaces with a 200 ppm chlorine solution for 24-48 hours is also recommended by Wyban and Sweeney (1991). This concentration of chlorine did not inactivate MBV within 24 hours. Therefore, a longer exposure time or higher chlorine concentration is required.

Shrimp hatcheries commonly use chlorine at 100-150 $mg \cdot l^{-1}$ for 30-60 minutes to disinfect tanks and pipes, and 50-100 $mg \cdot l^{-1}$ to treat building surfaces (Brock 1983; LeBlanc and Overstreet 1991). Our results indicate that chlorine concentrations currently recommended for disinfection in shrimp hatcheries are not sufficient to inactivate MBV. Treating hatchery tanks, equipment and surfaces with a solution of 1,000 $mg \cdot l^{-1}$ chlorine for 24 hours appears to be more reliable.

BP is inactivated by chlorine concentrations of 200 $mg \cdot l^{-1}$ and 1,600 $mg \cdot l^{-1}$ for 1 hour and 60 seconds, respectively (LeBlanc and Overstreet 1991). MBV was not inactivated by these treatments, suggesting that the occlusion body of the virus, which may represent a barrier to disinfection, is more resistant in MBV than in BP.

Bath stations for feet and equipment are used between hatchery buildings to prevent the spread of disease. An exposure to 1,600 $mg \cdot l^{-1}$ chlorine for 60 seconds did not inactivate MBV, indicating

that quick-rinse chlorine treatments are effective only if very high concentrations are used. Calcium hypochlorite is therefore not suitable as a footbath. The degrading effect of sunlight on chlorine would also reduce its effectiveness as a footbath, as foot stations are commonly positioned outside hatchery buildings.

The effectiveness of chlorine as a disinfectant may be reduced in water with a high organic load (Finlay 1978). Frerichs (1990) found proteinaecous host tissue reduced the level of chlorine available for virus inactivation. However, the absence of small amounts of host tissue in our experiments did not significantly increase the virucidal action of chlorine.

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