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Effect of different pH and salinity levels on the viability of *Penaeus monodon* baculovirus (MBV) in *Penaeus smisulcatus*

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Abstract: The virucidal effect of pH and salinity on *Penaeus monodon* baculovirus (MBV) was investigated by water borne inoculated methods exposing PL12 *Penaeus semisulcatus* for 10 hours and observing for 10 days at temperature 28-30°C. For this purpose, one thousand virus free PL12 of *P. semisulcatus* were divided into five groups and each group placed in a 10 L plastic basin containing chlorine–treated sea water at 35ppt. The salinity of basins water after 12 hours changed to 5, 10, 20, 30 and 40ppt using synthetic sea salt. To study the effect of pH, solution with various pH of 3, 5, 7, 8, 9 and 12 were prepared using 1N NaOH and 1N HCL to Na_2HPO_4 solution. For transmission of MBV, an aliquot of approximately 10 frozen infected larvae were used for each experiment by water born inoculated method. Ten hours after inoculation, the shrimp were removed from jar and introduced into another jar with the same salinity or pH. During 10 days experiment, the rate of infection (ROI), severity of infection (SOI) and accumulative mortality were varied among the MBV-infected. *Penaeus mondon* baculovirus was able to infect *Penaeus semisulcatus* at salinity ranging from 5-40ppt whereas at pH 3 and 12, MBV was completely inactivated. MBV was not able to infect larvae at pH 5, 7, 8 and 9.

Keywords: *Penaeus monodon*, baculovirus, *Penaeus semisulcatus*, Rate of infection (ROI), Severity of infection (SOI), Accumulative mortality, pH, Salinity

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Introduction

Infection and disease caused by *Penaeus monodon* baculovirus (MBV) in both cultured and wild penaeid shrimps are influenced by environmental parameters. Consequently, manipulation of these parameters can control or eliminate infections, benefiting production of cultured penaeids. Salinity and pH are important water quality variables, but they also strongly affect other water quality variables (Boyd, 1989).

Extreme pH conditions of 1.2 and 12.4 reduced infectivity of *Heliothis* NPV (Ignoffo & Garcia, 1966), and heat inactivated cytoplasmic-polyhedrosis viruses of *Bombyx mori* (Arugue *et al.*, 1963) and *Colias eurytheme* (Tanada & Chang, 1968). Gudauskas and Canerday (1968) further demonstrated reduced infectivity of *Heliothis* NPV and *Trichoplusia ni* NPV when exposed to extreme acid or alkaline conditions, UV light, and heat. In contrast, little information is available about the effects of chemical and physical conditions on the infectivity of aquatic shrimp baculoviruses. Momoyama (1989a) inactivated baculoviral midgut gland necrosis (BMN) virus using UV light, heat and desiccation. Later papers by the same author (Momoyama 1989 b,c) demonstrated the tolerance of BMN to ether, NaCl, pH, freezing conditions, and seawater when free from its host.

LeBlanc and Overstreet (1991) reported that heat, UV light and pH can inactivate *baculovirus penaeid* (BP). They showed BP was completely inactivated within 30 min after exposure to pH 3, whereas exposure to pH 11 extended the prepotency period (period before infection is apparent) but did not inactivate the virus. Chen *et al.* (1992) used formalin and iodophor to eliminate *P. monodon* baculovirus (MBV) from nauplii and fertilized eggs.

Chang *et al.* (1998) investigated the virucidal effects of several physico-chemical factors besides pH, salinity and some chemical disinfection on white spot syndrome baculovirus (WSBV) by infectivity assay using juvenile black tiger

prawn, *Penaeus monodon*. WSBV was completely inactivated by high acidity, pH 1 for 10 min, pH 3 for 1 hr and by high alkalinity of pH 12 for 10 min at room temperature (25°C). In this study the effect of pH and salinity as a disinfectant for inactivation of MBV in *P. semisulcatus* is investigated.

Materials and Methods

To determine the effect of salinity on the occurrence of MBV disease, one thousand virus free PLs 12 of *P. semisulcatus* were divided into five groups; they were checked by wet mount and PCR for MBV and then used in the experiment. Each group was placed in a 10 L plastic basin containing chlorine-treated seawater at 35ppt salinity. The salinity of basins was subsequently changed to 5, 10, 20, 30 and 40ppt using synthetic sea salt (Merck). The acclimation of PLs was carried out by floating the plastic bag containing PLs for 30 minutes in the plastic basins with the water temperature around 28-30°C, pH between 6-7 and the same salinity in the bag. After acclimation, 120 larvae were removed from each basin and divided into three subgroups and placed into 1.5 L aerated glass jar as replicate. Each jar contained 1 L of chlorine–treated seawater with the same salinity as the original container.

For transmission of MBV, an aliquot of approximately 10 frozen infected larvae of *Peneaus monodon* that showed the MBV infection by wet mount and PCR with adding 2 mL seawater (32ppt) was used for each experiment. The shrimp were thawed, homogenised with a tissue grinder and placed in an acid washed 5 cm diameter glass petri dish. The thawed homogenized aliquot of viral infected larvae was divided into three portions and each was introduced into one of the experimental glass jars. Ten hours after the inoculation process, the animals were removed from glass jars and were rinsed with chlorine-treated seawater with the same salinity for 10 minutes before introducing into another jar with chlorine-treated seawater with

the same salinity.

Water was exchanged daily in all jars to prevent salinity changes and accumulation of waste products and bacteria. To avoid contamination among experimental groups and among replicates, the contaminated water was carefully discarded and sieves, forceps, glass wares, pipettes and hands were disinfected with 100ppm chlorine solution (BDH Laboratory supplies, about 35% active ingredient) for 10 minutes after exposure to larvae and contaminated water.

Negative controls (not exposed to virus) for each salinity level were maintained in the same manner as their exposed counter parts. The unexposed control groups were kept in a separate room in order to avoid any possible contamination.

All shrimp groups were maintained on a diet of newly hatched *Artemia salina* nauplii three times per day at a rate of 1mL (with approximately 500 *Artemia* per mL) per jar. The water temperature in all jars ranged from 28-30°C, pH 8 -8.3 and dissolved oxygen between 5.9 - 6.3 mg/L.

For a period of 10 days, after each water exchange, the number of PLs in all jars were counted and recorded daily. For histology, four larvae were sampled from each jar, two of them were fixed in Davidson's fixative for 24 hours for light microscope and two others were fixed in glutaraldehyde fixative for electron microscope studies as described by Lightner (1996) methods.

To study the effect of pH on the viability of MBV, solution with various pH (3, 5, 7, 8, 9, and 12) were prepared with addition of appropriate amount of 1N NaOH (Merck) and 1N HCL (Merck), to 0.2 M Na_2HPO_4 (Merck) or 0.2 M NaH_2PO_4 (Merck) solutions. Each aliquot of infected larvae was processed in the way as described earlier and was then pipetted into 9mL of each 0.2 M sodium phosphate buffer with different pH. Viral homogenate buffer mixtures as well as unbuffered virus in seawater were incubated at room temperature (25°C) for 2 hr. After the incubation period, supernatant was removed and the precipitate was rehydrated with 35ppt seawater and each of them divided into three portions, which in turn

were introduced into three replicates set-up in aerated 1.5 L glass jars. Each jar contained 1 L of chlorine–treated seawater and 40 virus free PL–12 stage of *P. semisulcatus*. After 10 hours of the inoculation process, the animals were removed from glass jars and were rinsed with chlorine treated seawater for 10 minutes before introducing into another jar with chlorine–treated water. Water was changed daily in all jars to prevent accumulation of waste products and bacteria. To avoid contamination among experimental groups and among replicates, the contaminated water was carefully discarded and sieves, forceps, glassware's, pipettes and hands were disinfected as described earlier.

The negative control and sampling procedures were similar to those described earlier. All shrimp groups were maintained on a diet of newly–hatched *Artemia salina* nauplii as described earlier and sampling for histology was done as described earlier.

Estimation of prevalence was calculated using the formula as follows (Natividad & Lightner, 1992):

$$\text{ROI (or Prevalence)} = \frac{\text{Number of infected shrimp}}{\text{Total number of shrimp examined}} \times 100$$

All samples fixed in Davidson's fixative for 24 hr were processed by routine histopathology using hematoxylin-eosin/phloxine stains (Bell & Lightner, 1988). Diagnosis of MBV infections in the test shrimp was done by using Lightner's methods (1983). The severity of infection (SOI) description based on the numerical value system of 0 to 4 grades was first applied by Lightner *et al.* (1983).

Analysis of Variance (one way ANOVA) using SPSS Statistical software (Version 10.0.1) was used in the analysis of the accumulative mortality among infected and control groups and (two way ANOVA) used in the analysis of SOI and ROI at different concentrations of pH and salinity.

Results

The results of percentage of infection (ROI) of PL 12 *P. semisulcatus* with MBV at different concentration of salinity has shown in Table 1. On the first day shrimp did not show any signs of infection at salinity 5-40ppt. The Table shows sixty percent ROI value on second day for 10ppt salinity. On day 4 at salinity 20ppt, ROI was 40% and 100 % ROI was seen on the sixth day. On the eighth day all shrimp were dead at 20 ppt salinity. At salinity 30 and 40 ppt, 40% and 20% ROI was observed on the second day, respectively, and 100% ROI observed on the sixth day and eighth day, respectively. The control jar at different salinities did not show any signs of infection during the experiment.

There was no significant difference ($P>0.05$) in the severity of infection (Table 2 and Fig. 1) between the infectivity of MBV in larvae maintained at different salinity concentrations and the control groups. Larvae maintained at salinity 5ppt did not show any grade of infection (grade 0) and all shrimp were dead within 2 days. The percentage of infection at 10ppt, 20ppt, 30ppt and 40ppt of salinity during the experiments were light and moderate, and severity of infection was similar in all the experiment groups. The control groups did not show signs of infection.

Figure 3 shows the average accumulative mortality at different salinities and there was no statistical significance ($P>0.05$) among the infected and control groups of larvae maintained at salinities 5, 10 and 20ppt. However, there was significant difference ($P<0.05$) among the infected and control groups larvae at 30 and 40ppt. The average accumulative mortality was highest (75%) at salinity 5ppt among the infected groups and the control jar at this salinity showed (69%) accumulative mortality. The lowest accumulative mortality rate was observed at 40ppt salinity (62.5%) and the unexposed control group at this concentration showed the lowest (31.5%) accumulative mortality.

Table 3 shows the rate of infection at different pH concentrations. MBV was completely inactivated at pH 3 and shrimp examined at this salinity did not show any signs of infection during the experiment. At pH 5, 20% and 60% ROI was observed on fourth day and sixth day, respectively and on the eight day all shrimp were dead. At pH 7, 8 and 9, 100% ROI was observed on the 6th day after which all shrimp were dead. At pH 12, mild infection was observed on the sixth day and the ROI was 10%. The pH 12 was highly effective in inactivating the MBV virus. At pH 7, 8 and 9, there was progressive increase of ROI with increase of age after inoculation. None of the control jars showed any sign of infection during the experimental period.

Severity of infection expressed as grade of infection (Table 4 and Figure 2) showed that MBV infectivity was reduced at pH 3 and at pH 12 in which case the shrimp showed light infectivity of grade 1, 5%, while the pH 3 did not show grade of infection (100% grade 0). None of the control jars showed any sign of infection during the experiment at period.

Figure 4 shows the accumulative mortality at different pH concentrations. The lowest average accumulative mortality was observed at pH 3 (25%) and the unexposed control group at this concentration showed an average of 19.93% accumulative mortality during the experiment. The accumulative mortality amongst pH 3 and 12 and all other pH examined were significantly different ($P<0.05$). Higher accumulative mortality was observed at pH 9 when the exposed group showed 63.42% accumulative mortality whilst the control group showed 23% accumulative mortality.

Table 1: Percentage of infection (ROI) in PL12 of *P. semisulcatus* infected with MBV at different concentrations of salinities.

Salinity / Days	5		10		20		30		40	
	Inf	Con	Inf	Con	Inf	Con	Inf	Con	Inf	Con
1-DI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-DI	0.0	0.0	60.0	0.0	20.0	0.0	40.0	0.0	20.0	0.0
4-DI	-	-	100.0	0.0	40.0	0.0	60.0	0.0	60.0	0.0
6-DI	-	-	-	-	100.0	0.0	100.0	0.0	80.0	0.0
8-DI	-	-	-	-	-	0.0	100.0	0.0	100.0	0.0
10-DI	-	-	-	-	-	-	-	-	-	-

Con: control **Inf: infection** **DI: Day of infection** **PI: Post infection**

Table 2: Severity of infection (expressed as grade of infection) of MBV in PL12 of *P. semisulcatus* exposed to different concentrations of salinities

Salinity / Days	5		10		20		30		40	
	Inf	Con	Inf	Con	Inf	Con	Inf	Con	Inf	Con
1-DI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-DI	0.0	0.0	2.0	0.0	2.0	0.0	2.0	0.0	2.0	0.0
4-DI	-	-	3.0	0.0	3.0	0.0	3.0	0.0	3.0	0.0
6-DI	-	-	-	-	3.0	0.0	3.0	0.0	4.0	0.0
8-DI	-	-	-	-	-	0.0	3.0	0.0	4.0	0.0
10-DI	-	-	-	-	-	-	-	-	-	-

Con: control **Inf: infection** **DI: Day of infection** **PI: Post infection**

Table 3: Percentage of infection (ROI) in PL 12 of *P. semisulcatus* infected with MBV at different pH concentrations

pH / Days	3		5		7		8		9		12	
	Inf	Con	Inf	Con	Inf	Con	Inf	Con	Inf	Con	Inf	Con
1-DI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-DI	0.0	0.0	0.0	0.0	40.0	0.0	40.0	0.0	80.0	0.0	0.0	0.0
4-DI	0.0	0.0	20.0	0.0	80.0	0.0	80.0	0.0	100.0	0.0	0.0	0.0
6-DI	0.0	0.0	60.0	0.0	100.0	0.0	100.0	0.0	-	0.0	0.0	0.0
8-DI	0.0	0.0	100.0	0.0	-	0.0	-	0.0	-	0.0	10.0	0.0
10-DI	0.0	0.0	-	0.0	-	0.0	-	0.0	-	0.0	100.0	0.0

Con: control **Inf: infection** **DI: Day of infection** **PI: Post infection**

Table 4: Severity of infection (expressed as grade of infection) of MBV in PL 12 of *P. semisulcatus* exposed to different pH concentrations

pH / Days	3		5		7		8		9		12	
	Inf	Con	Inf	Con	Inf	Con	Inf	Con	Inf	Con	Inf	Con
1-DI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-DI	0.0	0.0	1.0	0.0	2.0	0.0	2.0	0.0	2.0	0.0	0.0	0.0
4-DI	0.0	0.0	2.0	0.0	3.0	0.0	3.0	0.0	3.0	0.0	0.0	0.0
6-DI	0.0	0.0	3.0	0.0	3.0	0.0	3.0	0.0	4.0	0.0	0.0	0.0
8-DI	0.0	0.0	-	0.0	-	0.0	-	0.0	-	0.0	1.0	0.0
10-DI	0.0	0.0	-	0.0	-	0.0	-	0.0	-	0.0	1.0	0.0

Con: control **Inf: infection** **DI: Day of infection** **PI: Post infection**

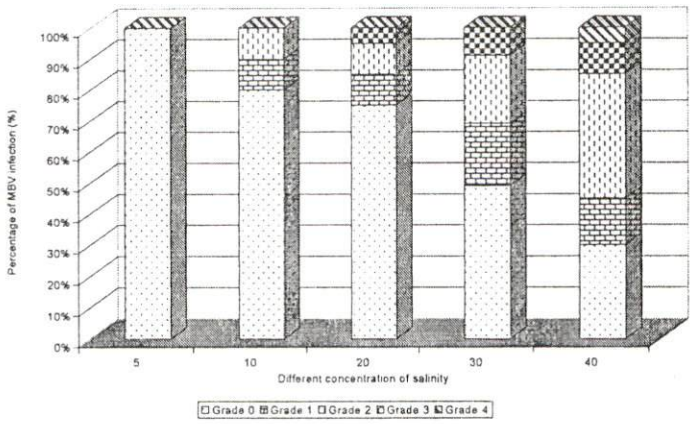

Figure 1: Effect of salinity on MBV infectivity in PL 12 of *P. semisulcatus*. The shrimp were analysed after 6 days post-exposure

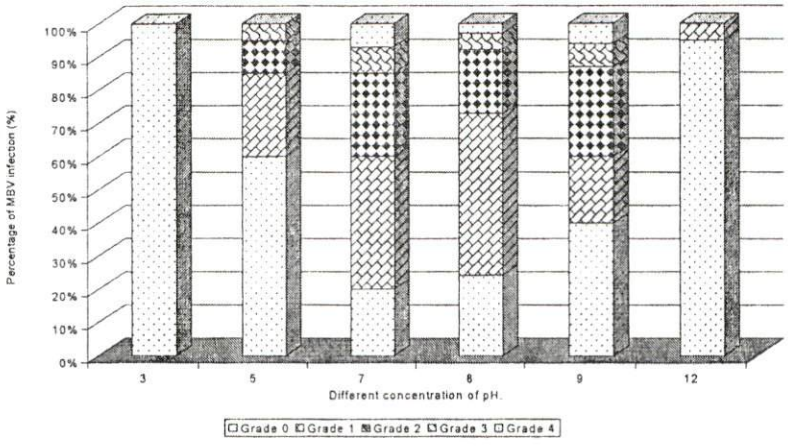

Figure 2: Effect of pH on MBV infectivity in PL 12 of *P. semisulcatus*. The shrimp were analysed after 6 day post-exposure

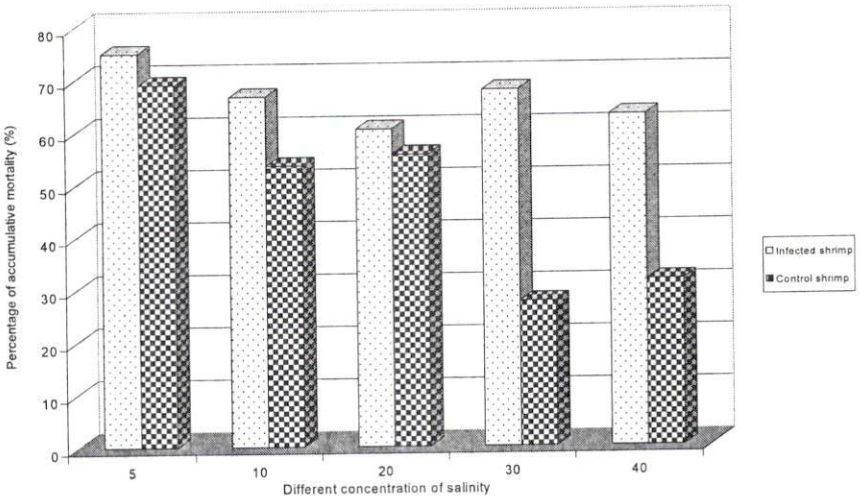

Figure 3: Accumulative mortality in PL 12 of *P. semiculcatus* infected with MBV and the control groups maintained at different salinities

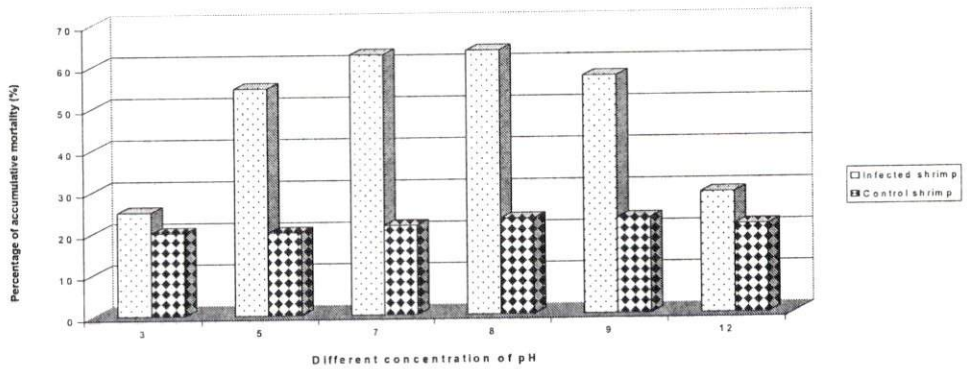

Figure 4: Accumulative mortality in PL 12 of *P. semisulcatus* infected with MBV and the control groups maintained at different pH

Discussion

Results of this study showed that MBV survived in salinities from 5ppt to 40ppt and could infect the shrimp that were maintained and exposed to this range of salinities. Our findings also showed the shrimp maintained at salinity of 5 ppt were all dead on the second day. Mortalities of *P. semisulcatus* at low salinities ranging from 5 to 15ppt has also been reported by Van Zaling (1984). Mortalities could not have occurred due to MBV infection because of the followings: 1) virus was not detected by histopathology thecnique, 2) Tokhmafshan (2001) showed that virus took at least 2 days to infect the host, and 3) even the control shrimp which were not exposed to MBV challenge were all dead on day 2. This indicated that the shrimp died due to the low salinity.

Shrimp at salinities of 20ppt, 30ppt and 40ppt when exposed to MBV, survived until seventh, eighth and fifth day, respectively. On the other hand, control shrimps showed that the shrimp survived in similar concentration until the end of experiment i.e. 10 days. In the present study, however, the shrimps were held at low salinities for 10 hr, during the infectivity assay and after that for long time. In this case though the virus was still alive, it was possible that the particular receptors for MBV on shrimp cell surface were changed in low salinity water, resulting in the failure of viral entrance into the shrimp cells and thus no infection occurred. Appropriate cell surface receptor is an essential prerequisite for a virus in a host cell (Voyles, 1993).

Several researchers have studied the effect of different salinities on the infectivity of baculovirus. Chang *et al.* (1998) reported that sodium chloride in concentrations ranging from 0% to 10% had no virucidal effect on the infectivity of WSBV. However, lower salinity has been reported to prevent the occurrence of white spot syndrome in Taiwan and Thailand (Chen H.C., cited in Chang *et al.*, 1998). Momoyama (1992) studied inactivation of BMNV by chemical and physical factors and showed that BMNV was inactivated by 25% NaCL within 10 hr and

12.5% NaCL within 24 hr. Wang (2000) reported that lower salinity (0 and 2.5ppt) could successfully prevent WSSV infection or at least reduced the severity of the disease. The difference between the results of Chang *et al.* (1998), Momoyama (1992) and Wang (2000) and those observed in our study on MBV may be due to the difference in the virus species tested, methods of infectivity assay and susceptibility of the different stages of shrimp used for the study. Lowering of salinity may not be appropriate to eradicate MBV during a culture cycle in a hatchery or a growout systems as it will stress the animals and cause severe mortalities in *P. semisulcatus*. However, based on the present studies, low salinities could be used to eradicate MBV. This could be effectively applied to empty ponds or hatchery tanks. If necessary, this also could be done with combination of other disinfectants.

Cultured shrimps are very sensitive to the pH variation of the water. The pH of a typical culture pond usually ranges from 7.2 to 9.0 and the most suitable pH for shrimp ranges from 7.8 to 8.3 (Jean, 1990). In the present study MBV was completely inactivated at pH 3, and at pH 12 the activity of MBV was reduced by 95%. The infectivity of MBV was not affected at pH 5, 7, 8 and 9. Acidic conditions of pH 2 has also been reported to affect insect viruses (Ignoffo & Garcia, 1966 ; Gudauskas & Canerday, 1968).

Our finding on MBV are similar to those of Momoyama (1989b), who demonstrated that BMN was infective when shrimp were maintained at pH 3 for 3 hr, but were not infective at pH 10. Summers (1977) also reported that *B. penaei* could not be inactivated at pH 11 for 6 hr. Granados and Williams (1986) reported occluded virions of *B. penaei* to be stable insect viruses over a broad range of pH. These tested shrimp and insect NPVs apparently remain highly infective at any pH ranging from 4 to at least 11. Because of baculoviridae tolerance to a broad range of pH, the disinfections in aquaculture system using pH as a parameter, is probably not economically feasible. However, further study of the role of pH and its effects

on MBV could better define the mechanisms affecting shrimp MBV infections.

There was no significant difference in the data on shrimp exposed to MBV infection at pH 5, 7, 8 and 9. In these cases virus was stable and occlusion bodies were observed after the second days and the shrimp died from day 3 onwards.

The findings of this study are consistent with Chang *et al.* (1998) who reported that WSBV was stable at pH 8 but its infectivity apparently decreased by exposure to pH 3 for 10 min. It was rendered completely non-infectious at pH 1 or pH 12 for 10 min. BMNV and BP, on the other hand, can tolerate alkaline conditions as high as pH 13 and pH 11 (Momoyama, 1989b ; LeBlanc & Overstreet, 1991). Chang *et al.* (1998) concluded that both extremely acidic and alkaline conditions could render WSBV non-infectious. They suggested extremely acidic or alkaline solutions (pH 1 and pH 12) could be used to wash equipment that is tolerant to strong acids and bases, such as glassware, some plastic containers, and nylon nets. Treating the bottom soil with lime can produce an alkaline environment (about pH 12) which would effectively inactivate baculoviruses such as WSBV and MBV before the shrimps are stocked. The use of pH alteration as a control measure in shrimp pond during culture period is limited, as the optimum pH level for penaeid is between 7.5 and 8.3 (Chanratchakool *et al.*, 1993).

When comparing the baculoviruses in fish, our results are dissimilar with Whipple and Rohovec (1994) who studied the effect of low pH on selected viral and bacterial fish pathogen. They showed the infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) were suspended in buffered medium at pH 7 or 4, or in fish silage at pH 4 and survival at selected pH was determined. Arimoto *et al.* (1996) reported that the striped jack nervous necrosis virus (SJNNV) was inactivated at pH 12, but not at pH 3 or pH 7. They recommended that rubber shoes, plastic buckets and nylon nets must be washed with an alkaline solution (pH 12) for the inactivation of SJNNV and then neutralized it with diluted hydrochloride solution.

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