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Efficacy of Calcium Hypochlorite on the Prevalence of Microsporidiosis (*Thelohania*) in Pond-Reared *Litopenaeus vannamei*

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ABSTRACT

The efficacy of calcium hypochlorite for controlling microsporidian (*Thelohania* or *Agmasoma*) infection by treating the water before stocking Pacific white shrimp, *Litopenaeus vannamei*, postlarvae (PL) was studied in intensively cultured ponds. Five ponds (of 4 rai each) were used as the treatment group in which calcium hypochlorite at a concentration of 18 mg/l was added into the ponds while all the aerators were operating. In the control group, no calcium hypochlorite was used. PL10 were stocked at a density of 80 PL/m^2 and shrimp were fed with commercial pelleted feed throughout the 120-day culture period. Results showed that the prevalence of microsporidian (*Thelohania, Agmasoma*) infection of shrimp in the treatment group was significantly lower than that of the control group ($P<0.05$). At day 50, the average highest percentage of microsporidian infection in the treatment ponds was 5.40% compared with 25.16% in the control ponds. The infected shrimp gradually died off or were eaten by the healthy shrimp so that after harvesting only a few infected shrimp were found. In conclusion, in shrimp culture areas where microsporidian outbreaks previously occurred, calcium hypochlorite at the concentration of 18 mg/l should be used for water treatment before stocking the PL.

Key words: microsporidian, *Thelohania, Agmasoma*, *Litopenaeus vannamei,* calcium hypochlorite

INTRODUCTION

Since the Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand, allowed the introduction of specific pathogen-free (SPF) Pacific white shrimp (*Litopenaeus vannamei*) into Thailand for commercial culture in 2002, the production of Pacific white shrimp from intensive culture has increased tremendously.

In spite of the impact of major viral diseases such as white spot syndrome virus (WSSV), Taura syndrome virus (TSV) and yellow head virus (YHV) which caused heavy losses in some culture areas (Limsuwan, 2003; Limsuwan and Chanratchakool, 2004), the production of white shrimp in 2006 reached 500,000 metric tons. In other diseases such as microsporidiosis caused by microsporidian parasites, even though the mortality rate was not severe, infected shrimp had a whitish or milky discoloration of abdominal muscles which caused a marketing problem resulting in economic losses for the shrimp farmers. High prevalence rates of microsporidiosis in wild shrimp populations have been reported and linked to serious impacts on commercial fisheries (Lightner, 1996). Most microsporidian infections have been reported from the Americas

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(Iversen and Manning, 1959; Baxter *et al*., 1970; Feigenbaum, 1975). In Asia, microsporidiosis has been reported in cultured *P. monodon* from Malaysia (Anderson *et al*., 1989) and Thailand (Limsuwan, 1991; Flegel *et al*., 1992). Microsporidians from infected banana shrimp *(P. merguiensis*) as well as *P. monodon* were identified as *Agmasoma* (*Thelohania*) *penaei* (Flegel *et al*., 1992). It has been suggested that certain fish species act as intermediate hosts. At present, effective methods of treating microsporidiosis in shrimp have not been developed. Elimination of possible fish intermediate hosts before stocking of postlarvae (PL) may reduce the transmission of microsporidians in shrimp culture. Calcium hypochlorite ($Ca(OCl)_2$) is an effective chemical routinely used for water treatment before stocking the PL to prevent major viral diseases (Chitmon, 1995). The aim of this study was to evaluate the efficacy of calcium hypochlorite as a water treatment prior to stocking PL for preventing microsporidiosis in intensive culture of Pacific white shrimp.

MATERIALS AND METHODS

This investigation was conducted in a shrimp farm located in Nakhon Si Thammarat province, southern Thailand. Microsporidian infections have been reported in cultured *P. monodon* in this farm as well as in wild *P. merguiensis* in the surrounding culture areas. Ten growout ponds of approximately 4 rai (6,400 m^2) and two reservoir ponds of the same size were used in this study. Each pond was covered with polyethylene on the dike and down to the feeding area except in the middle of the pond where the sediments and sludge accumulate during the culture period. The salinity during the study period was 25-30 parts per thousand (ppt).

Water preparation and culture techniques

After the shrimp were harvested, routine pond preparation was carried out by removing the sludge from the middle of the pond. Water from the reservoir ponds allowed to rest for at least two weeks was pumped into 10 ponds until reaching a 1.5 m depth. Calcium hypochlorite (60% active ingredient), routinely used for elimination of wild crustaceans and other aquatic animals, was added into five ponds at a concentration of 18 mg/l. This concentration is commonly used for water treatment for nursing shrimp larvae and eradication of possible viral carriers of WSSV and YHV (Limsuwan, 2000). All the aerators were used for thorough mixing the chlorine in the ponds. For the five control ponds, no calcium hypochlorite was added and only the aerators were operated. One week later, inorganic fertilizer and cooked rice bran were added into the ponds in order to increase the phytoplankton and natural food to a suitable level which was measured by Secchi disc reading to obtain a transparency of 50-60 cm. This natural food preparation process took one additional week. SPF PL 10 determined to be free from WSSV, TSV and YHV by polymerase chain reaction (PCR) assays, were stocked into each pond at a density of 80 PL/m^2. Commercial pelleted feed was provided throughout the culture period. No water was exchanged during the first 40-50 days. More water exchange was required to maintain optimal water quality particularly during one month before harvesting the shrimp.

Infection rate during the culture period

Shrimp were observed for clinical signs of microsporidian infection throughout the 120-day culture period. During the first 29 days after stocking the PL, only shrimp from the feeding trays were observed daily. At day 30, shrimp were first sampled by the cast net and then sampled every 10 days until the shrimp were harvested. For each sampling time, at least 600 shrimp were randomly sampled from three different areas in the pond. The numbers of microsporidian-infected shrimp and the gross signs were recorded. The percentages

of microsporidian infection in the control and treatment ponds were statistically compared using Student's t-test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Clinical signs

The Pacific white shrimp were first observed to be infected with microsporidian parasites 15 days after stocking the PL into the growout ponds. In some ponds infected white shrimp were first found at day 20. Affected shrimp had a whitish or milky body appearance. When the shrimp became larger, this clinical sign was more easily observed especially dorsally from the cephalothorax to the middle of the body. However, some infected shrimp had a whitish appearance near the last segment of abdominal muscle (Figure 1). Internal gross signs of infected shrimp revealed that the white masses of microsporidian parasites replaced the striated muscles as well as other tissue types, including connective tissues and the hepatopancreas (Figure 2). Light microscopic examination of fresh specimens revealed that each sporophorous vesicle contained eight spores belonging to the genus *Thelohania* or *Agmasoma* (Figure 3) similar to microsporidians previously reported in *P. monodon* by Limsuwan (1991) and Flegel *et al.* (1992).

Prevalence and severity of the disease

The prevalence of microsporidian infection in Pacific white shrimp in the control and treatment ponds is shown in Table 1. The highest prevalence of infection reached 25.16 % in the control pond and 5.40 % in the treatment pond, which had a statistically significant difference ($P<0.05$). The highest average percentage of infection was found between 40-50 days in both groups. Subsequently, the prevalence of infection decreased slightly until day 80 and then sharply declined until day 100. After the shrimp were harvested, it was determined that only 3.42% of diseased shrimp remained from the control pond and less than 1% in the treatment pond. After this microsporidian infection was first observed in the white shrimp, the numbers of infected shrimp were observed to increase until

Figure 1 White shrimp heavily infected with microsporidian had a whitish or milky appearance of the body.

40-50 days. It was possibly because in the larger shrimp, the microsporidian parasites replaced more striated muscle and other tissues, so that the whitish appearance was more easily noticed in the larger shrimp than in the smaller ones. It was interesting that the percentage of infected shrimp found in the feeding trays increased during most of the culture period. Shrimp heavily infected with microsporidians had loose shells and grew slower than the healthy shrimp. The percentages of infected shrimp in the feeding trays and caught in the cast nets from different areas in the pond were also compared. Results clearly showed that the percentage of infected shrimp from the cast nets

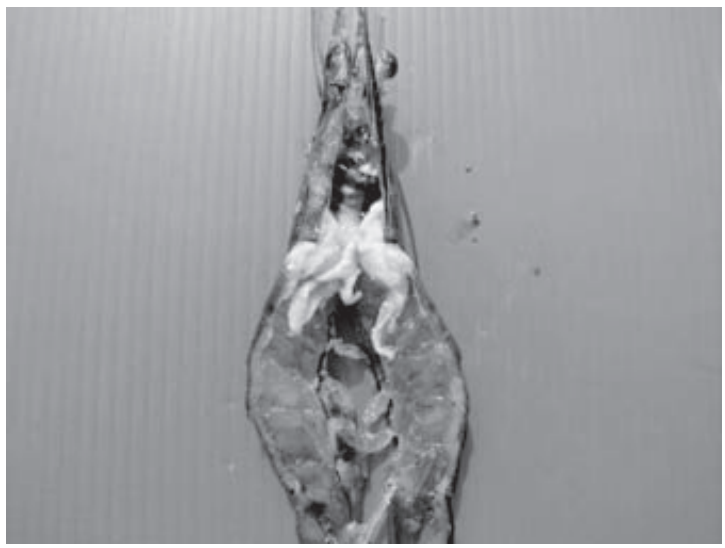

Figure 2 White masses of microsporidian replaced striated muscle and other tissues.

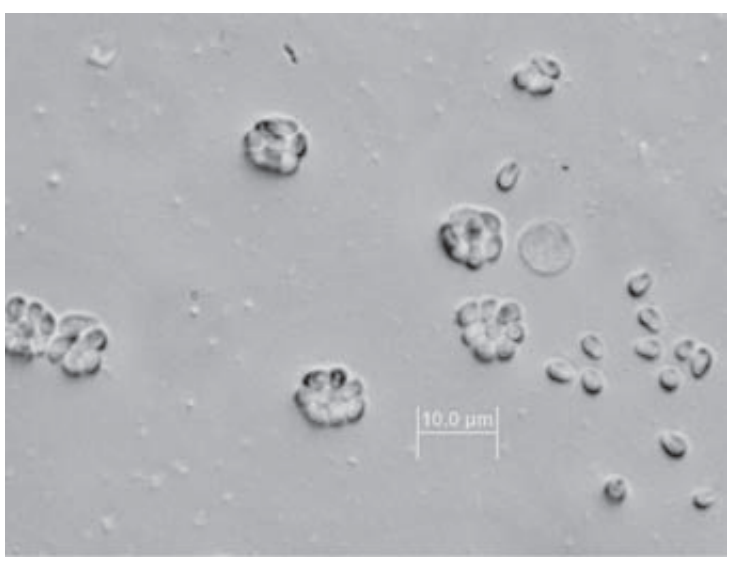

Figure 3 Unstained wet mounts of fresh specimens showed each sporophorous vesicle contained eight spores. (bar=10 mm).

was much lower than the number of infected shrimp found in the feeding trays. This was because the infected shrimp could not compete with the healthy shrimp during the feeding times and moved into the feeding trays for food where there were some feed samples. In this study, we used cast net samples for evaluating the percentages of infected and healthy of shrimp which gave more precise information than the observing the feeding trays.

In fact, the most severely infected shrimp after 80 days old had loose shells and were very weak due to the microsporidians having replaced most of the striated muscle tissues and hepatopancreases. After shrimp were 80-100 days old, the prevalence of infected shrimp from cast net sampling decreased because they gradually died off or were consumed by the healthy shrimp. At the time of harvest, only a few infected shrimp were found in both groups.

Diseased shrimp that survived until harvesting were smaller than the healthy ones. Moreover, after 50 days there were no newly microsporidian-infected shrimp, as no increase was found in the cast net samples and at harvesting no early clinical signs of infected shrimp were observed although untreated water from the reservoir was used for water exchange. Particularly, more water exchange was required when the shrimp became larger during the late culture period. Results from this study indicated that microsporidian infection occurred after the infective spore stages were eaten by the PL. Environmental conditions during PL stocking were more suitable for microsporidian infection of the PL than the water quality conditions after 50 days or because larger shrimp were more resistant to this microsporidian parasite. Calcium hypochlorite at the concentration of 18 mg/l used for water treatment had some effect on microsporidian spores as the prevalence of infection in the treatment ponds was significantly lower than that of the untreated control ponds. However, some microsporidian spores still survived despite exposure to chlorine water. Large amounts of sediments and organic matter might

Table 1 Percentage microsporidian infection of white shrimp from treatment and control ponds during the culture period of 120 days.

Experimental	Culture period (days)									
ponds	30	40	50	60	70	80	90	100	110	120
Treatment ponds										
1	1.3	4.5	5.0	4.2	4.1	3.9	2.4	1.6	1.3	0.6
2	3.6	5.2	5.6	5.3	4.7	4.4	2.7	1.5	1.2	0.9
3	2.3	4.7	6.1	5.8	5.4	3.8	2.3	1.3	1.0	0.7
4	2.5	5.5	5.2	4.9	4.7	3.5	2.1	1.1	0.9	0.8
5	1.6	5.3	5.1	4.6	3.9	3.8	1.8	1.2	1.1	0.5
Average	2.26[a]	5.04[a]	5.40[a]	4.96[a]	4.56[a]	3.88[a]	2.26[a]	1.34[a]	1.1[a]	0.7[a]
Control ponds										
1	15.6	23.4	25.1	23.4	21.2	19.1	14.6	8.2	6.4	4.7
2	14.4	26.2	25.6	22.3	20.8	18.8	13.1	6.4	5.3	3.8
3	17.3	27.6	27.3	25.1	23.7	21.4	16.2	7.6	4.5	2.6
4	15.1	20.7	24.1	22.7	20.9	17.7	13.8	6.5	4.7	3.4
5	12.7	24.8	23.7	21.1	19.7	18.2	14.4	7.8	4.3	2.6
Average	15.02[b]	24.51[b]	25.16[b]	22.92[b]	21.26[b]	19.04[b]	14.42[b]	7.3[b]	5.04[b]	3.42[b]

The different alphabets in the same column mean significant difference ($P<0.05$)

reduce the toxicity of chlorine as well as its efficacy to kill the susceptible stages of the parasite. A high pH value also decreased the toxicity of chlorine (Zillich, 1972; Floyd, 1979).

Microsporidians are not considered to be of great economic significance for cultured shrimp in comparison with viruses and bacteria, as the prevalence of infection typically reaches 10-20 % in cultured populations and mortality is not typical (Lightner, 1993). In this study, the prevalence of infection reached 25.16 % after 50-day post-stocking. Later during the culture period, despite the infected shrimp being eaten by healthy shrimp, the prevalence of infection did not increase. This suggested that transmission was unsuccessful when the healthy shrimp ate infected shrimp or were exposed to waterborne spores as reported by Iversen *et al*. (1987). Microsporidiosis was transmitted experimentally to *P. duorarum* PL by feeding them with the feces of spotted sea trout (*Cynoscion nebulosis*) which was fed by the infected shrimp (Iversen *et al*., 198G).

Flegel *et al*. (1992) observed that *A. penaei* was not transmitted horizontally in adolescent *P. monodon* and that vertical transmission from broodstock to eggs was unlikely. Pasharawipas and Flegel (1994) reported that two fish species, *Priacanthus tayenus* and *Scatophagus argus*, gave positive hybridization signals to a DNA probe for *A. penaei*. These two finfish species were suspected as being the intermediate hosts of this microsporidian parasite.

CONCLUSION

Calcium hypochlorite at the concentration of 18 mg/l commonly used for water treatment and eradication of wild crustaceans before stocking PL significantly reduced the prevalence of microsporidian infection. The highest prevalence infection was observed during 40-50 days post-stocking. The Infected shrimp developed opaque muscles especially dorsally from the cephalothorax to the middle of the body and gradually died off or were eaten by the healthy shrimp during 80-100 days of culture. At the time of harvest, only a few infected shrimp were found. Preventive measures for this microsporidian consisted of elimination of all possible intermediate hosts, especially fish species, in shrimp culture areas where outbreaks previously occurred before stocking the PL.

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