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Efficiency of Chitosan to *Vibrio* spp. Isolated From Diseased Black Tiger Shrimp, *Penaeus monodon* Fabricius in Thailand

Ong-ard Lawhavinit [1]*, Win Surachetpong[1], Benjamas Inthasri[1] and Nontawith Areechon[2]

ABSTRACT

Efficiency of chitosan to *Vibrio* spp. isolated from diseased black tiger shrimp was studied. The result showed that the Minimum Bactericidal Concentration (MBC) of chitosan is 1,000 ppm. The chitosan could be used to induce the bacteria both high and low organic matters. The effects of the inoculation size on the activities of chitosan at 1,000 ppm were 10^2 - 10^8 CFU/ml. The MBCs of chitosan tested with 47 isolated *Vibrio* spp. fell in the range of 1,000-10,000 ppm. The majority of the field isolated *Vibrio* spp. has retained susceptibility to chitosan at 1,000 ppm. In the safety test with 1 month black tiger shrimp, no shrimp died at 0.1-1.0% of chitosan but 50% experimental shrimp died at 2.0% chitosan in 96 hours. Base on the results, the 1.0% chitosan could safe use for prevention the luminescence vibriosis in black tiger shrimp culture.

Key word: black tiger shrimp, *Peneaus monodon*, chitosan, vibriosis

INTRODUCTION

Presently, the shrimp consumers are afraid of the antibiotic residue problem. Because, the farmer lack the knowledge use the antibiotics. Therefore, by convincing, the shrimp exporter and the farmers were loss a lot of money. Thai government is urgently solving this problem, the farmer to use the natural products instead of the antibiotics. But the farmers are no response. Because of, the most important bacterial disease in shrimp is vibriosis (Karunasagar *et al*., 1994). The farmer used the antibiotics to kill or to prevent the *Vibrio* spp. However, they know some natural products that can be use to control bacteria. Razdan and Pettersson (1994) reported chitosan could react with the cell wall and reduce the growth of bacteria. The chitosan is produced from chitin which is extracted from crab and shrimp shell (Roberts, 1992). Chitosan is a non-toxic, biocompatible and biodegradable natural polymer. The food, drugs, and cosmetic industry use the chitosan in a composition of their products. Therefore, chitosan should be safe for use to stop the bacterial diseases of shrimp.

The objective of this study was to investigate the efficiency of chitosan as control of *Vibrio* spp. from diseased black tiger shrimp, *Penaeus monodon* Fabricius in Thailand. The minimal bactericidal concentration of chitosan to the *Vibrio* spp. was studied here.

[1] Department of Veterinary Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand.

[2] Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand.

* Corresponding author, e-mail: fvetonl@ku.ac.th

MATERIALS AND METHODS

The *Vibrio* spp. uses in the test

The bacteria uses in this test were *Vibrio* spp., which were isolated from the diseased shrimp which were collected from some provinces in Thailand, such as Chanthaburi, Rayong, Prachin Buri, Chachoengsao, Bangkok, Phetchaburi, Prachuap Khiri Khan, Chumphon, Ranong, Krabi, Nakhon Si Thammarat, Trang, and Pattani. They were identified as *Vibrio alginolyticus*, *Vibrio cholaere*, *Vibrio damsela*, *Vibrio fluvialis*, *Vibrio harveyi* , *Vibrio parahaemolyticus Vibrio vulnificus* and *Vibrio* spp. with the API 20NE (Geiss *et al*., 1985). The bacteria were stocked in Thiosulfate citrate bile salt sucrose (TCBS) at ambient room temperature, during the experiments.

The chitosan was used in the test

The chitosan was obtained from the shrimp shop and was sent to check the purity at Department of Fishery Product, Faculty of Fisheries, Kasetsart University, Thailand. The results showed that, the chitosan concentration is 3.63 percent at the pH 4.57 by the method of turbidimeter A.O.A.C (1984).

Minimum bactericidal concentration (MBC) of chitosan

MBC of chitosan was done with the *Vibrio harveyi* and compared for all of 47 isolated of the bacteria, which received from Dr.Janenuj Wongtavatchai, Dr.Nontawith Areechon and AAHRI, Department of Fishery, Thailand. In this experiment, MBC was defined as the lowest concentration at which no bacterial growth was observed. The determination of MBC was conducted in Tryptic Soy broth (TSB) in 2% NaCl and 10% TSB in 2% NaCl (10ml per each tube) containing serially graded concentrations of chitosan (0, 25, 50, 100, 500, and 1,000 ppm). After inoculating (1ml of inoculated bacteria into serial dilution of chitosan), estimate viable bacteria cells were equal to 10^2 CFU/ml in the *V. harveyi* experiments and McFraland number 2 or 3×10^8 CFU (McFarland, 1907) in 47 isolated *Vibrio* spp. tested, respectively. All the tubes were incubated at ambient room temperature for 24 hours. The bacterial growth at MBC level was checked with streak on TCBS that were incubated at ambient room temperature for 24 hours. For data analysis, if no viable bacteria were observed at the lowest dilution, it is MBC of the isolated bacteria in this test.

The safety test of chitosan were done with one month black tiger shrimps

The 0.1, 1.0, 2.0 and 0 percent of chitosan were put into the culture black tiger shrimp water in 6 liters aerated aquarium. Ten pieces of 1 month black tiger shrimp were culture in 12 aquariums, 3 replication per treatments. The salinity of culture water was 20 parts per thousand. The culturing water quality, such as pH, temperature, alkalinity, salinity, ammonia and dissolved oxygen were checked at 0, 48, and 96 hours. The mortality of experimental shrimp was recorded and determined LD. 50 at 96 hours.

RESULT AND DISCUSSION

The results of chitosan' MBC for *Vibrio harveyi* isolated from the diseased black tiger shrimp is 1,000 ppm under the condition of the bacterial 10^2 CFU/ml in normal TSB and 10%TSB mixed with the 2%NaCl (Table 1 and 2). The effects of the inoculation size on the activities of chitosan at 1,000 ppm were 10^2 - 10^8 CFU/ml (Table 3). That mean, the chitosan could inhibit Vibrio *harveyi* under the high organic (normal TSB) and low organic concentration (10% TSB). Therefore, the chitosan could be used in shrimp culture for prevention or control the number of bacterial diseases in water culture. The used concentration is depending on the cost of chitosan

that will be studied further to compare with other products such as benzalkonium chloride and povidone iodine in the next step.

The susceptibility of field isolated *Vibrio* spp. was shown in Table 4. The MBCs of chitosan tested with 47 isolated *Vibrio* spp. fell in the range of 1,000-10,000 ppm (Table 5). The majority of the field isolated *Vibrio* spp. has retained susceptibility to chitosan at 1,000 ppm. Recently, the antimicrobial activity of chitosan has been

Table 1 The Effect of chitosan in TSB with 2% NaCl on the survival of *Vibrio harveyi* at 10^2 CFU/ml, isolated from disease black tiger shrimp.

Chitosan concentration (ppm)	Replication 1	Replication 2	Replication 3
10,000	-	-	-
5,000	-	-	-
1,000	-	-	-
500	+	+	+
100	+	+	+
50	+	+	+
25	+	+	+
12.5	+	+	+
0	+	+	+

Note: +: growth of bacteria, -: no growth of bacteria. The *Vibrio harveyi* is received from AAHRI, Department of Fisheries, Ministry of Agriculture and Cooperative, Thailand.

Table 2 The Effect of chitosan in 10 %TSB with 2% NaCl on the survival of *Vibrio harveyi* at 10^2CFU/ml, isolated from disease black tiger shrimp.

Chitosan concentration (ppm)	Replication 1	Replication 2	Replication 3
1,000	-	-	-
500	+	+	+
100	+	+	+
50	+	+	+
25	+	+	+
0	+	+	+

Note: +: growth of bacteria, -: no growth of bacteria. The *Vibrio harveyi* is received from AAHRI, Department of Fisheries, Ministry of Agriculture and Cooperative, Thailand.

Table 3 Survival of various numbers *Vibrio harveyi* at the 1,000 ppm chitosan in 10% TSB with 2%NaCl after 24 hrs. at room temperature in TCBS.

Concentration of *Vibrio harveyi* (CFU/ml)	Replication 1	Replication 2	Replication 3
10^8	-	-	-
10^6	-	-	-
10^4	-	-	-
10^2	-	-	-

Note: +: growth of bacteria, -: no growth of bacteria. The *Vibrio harveyi* is received from AAHRI, Department of Fisheries, Ministry of Agriculture and Cooperative, Thailand.

Table 4 Number of 47 isolated *Vibrio* spp. from disease black tiger shrimp in Thailand, which were used in this experiment.

Genus species	Number of isolates
Vibrio alginolyticus	4
Vibrio cholaere	2
Vibrio damsela	1
Vibrio fluvialis	4
Vibrio harveyi	9
Vibrio parahaemolyticus	16
Vibrio vulnificus	1
Vibrio spp	10
Total	47

studied extensively, it has been shown that chitosan acts by disrupting the barrier properties of the outer membrane of gram negative bacteria (Ohtakara *et al*., 1988). The minimum inhibitory concentration (MIC) of chitosan ranged from 0.05% to more than 0.1% depend on the examined gram negative bacteria which were *Escherichia coli*, *Pseudomonas fluorescens*, *Salmonella typhimurium* and *Vibrio parahaemolyticus* (No *et al*., 2002). Our study confirms and supports the earlier findings regarding usefulness of chitosan as an antimicrobial activity. Based on the results described above, the chitosan exhibits potent inhibiting against the *Vibrio* spp. encountered in shrimp culture and to some extent against *Vibrio harveyi*. Chitosan is safe for used because it is used in the food, cosmetic, and drug industry and some environmental works use the chitosan to fix toxic substances such as heavy metal in water treatment. Actually, In the safety test with 1 month black tiger shrimp, no shrimp died at 0.1-1.0% of chitosan but 50% experimental shrimp died at 2.0% chitosan in 96 hours (Table 6). The water qualities were showed in Table 7. Base on the results, the 1.0% chitosan should be safe to use for prevention the luminescence vibriosis in black tiger shrimp culture.

In recent years the epidemic *Vibrio harveyi* disease of cultured black tiger shrimp has caused great economic damage to the farmers. And now, the consumers were aware of the antibiotic residue problem in the shrimp. Thai Department of Fisheries is strictly checked before giving the certificates document and tried to produce the Q shrimp product of Thailand. Under this condition, the chitosan is suitable as a replacement for antibiotic drugs in black tiger shrimp culture.

CONCLUSIONS

Presently, the shrimp consumers are afraid of the antibiotic residue antibiotic problem. Because, the farmer lack the knowledge to use the antibiotics. However, they know some natural products that can be use to control bacteria. The chitosan could react with the cell wall and reduce the growth of bacteria. The chitosan is produced from chitin which is extracted from crab and shrimp and is a non-toxic, biocompatible and biodegradable natural polymer. The food, drugs, and cosmetic industry use the chitosan in a composition of their products. Therefore, efficiency of chitosan to *Vibrio* spp. isolated from diseased black tiger shrimp was studied. The result showed that the MBC of chitosan is 1,000 ppm. The chitosan could be used for induce the bacteria both high and low organic matters. The effects of the inoculation size on the activities of chitosan at 1,000 ppm were 10^2 - 10^8 CFU/ml. The MBCs of chitosan tested with 47 isolated *Vibrio* spp. fell in the range of 1,000-10,000 ppm. The majority of the field isolated *Vibrio* spp. has retained susceptibility to chitosan at 1,000 ppm. In the safety test with 1 month black tiger shrimp, no shrimp died at 0.1-1.0% of chitosan but 50% experimental shrimp died at 2.0% chitosan in 96 hours. Base on the results, the 1.0% chitosan could safe to use for prevention the luminescence vibriosis in black tiger shrimp culture.

Table 5 Survival of isolated *Vibrio* spp. from disease black tiger shrimp at 500 1,000 and 10,000 ppm chitosan in 2%NaCl after 24 hrs inoculation at room temperature in TCBS.

No.	Genus species	Concentration of chitosan (ppm.)		
		500	1,000	10,000
1	*Vibrio alginolyticus*	+	-	-
2	*Vibrio alginolyticus*	+	-	-
3	*Vibrio alginolyticus*	+	-	-
4	*Vibrio alginolyticus*	+	-	-
5	*Vibrio cholaere*	-	-	-
6	*Vibrio cholaere*	-	-	-
7	*Vibrio damsela*	-	-	-
8	*Vibrio fluvialis*	+	-	-
9	*Vibrio fluvialis*	+	-	-
10	*Vibrio fluvialis*	+	-	-
11	*Vibrio fluvialis*	+	-	-
12	*Vibrio harveyi*	+	-	-
13	*Vibrio harveyi*	+	-	-
14	*Vibrio harveyi*	+	-	-
15	*Vibrio harveyi*	+	-	-
16	*Vibrio harveyi*	+	-	-
17	*Vibrio harveyi*	+	-	-
18	*Vibrio harveyi*	+	-	-
19	*Vibrio harveyi*	+	-	-
20	*Vibrio harveyi*	+	-	-
21	*Vibrio parahaemolyticus*	+	-	-
22	*Vibrio parahaemolyticus*	+	-	-
23	*Vibrio parahaemolyticus*	+	-	-
24	*Vibrio parahaemolyticus*	-	+	-
25	*Vibrio parahaemolyticus*	+	-	-
26	*Vibrio parahaemolyticus*	+	-	-
27	*Vibrio parahaemolyticus*	+	-	-
28	*Vibrio parahaemolyticus*	+	+	-
29	*Vibrio parahaemolyticus*	+	+	-
30	*Vibrio parahaemolyticus*	+	-	-
31	*Vibrio parahaemolyticus*	+	-	-
32	*Vibrio parahaemolyticus*	+	-	-
33	*Vibrio parahaemolyticus*	+	-	-
34	*Vibrio parahaemolyticus*	+	-	-
35	*Vibrio parahaemolyticus*	+	-	-
36	*Vibrio parahaemolyticus*	+	-	-
37	*Vibrio vulnificus*	+	+	-
38	*Vibrio* spp	+	-	-
39	*Vibrio* spp	-	-	-
40	*Vibrio* spp	+	-	-
41	*Vibrio* spp	+	-	-
42	*Vibrio* spp	+	-	-
43	*Vibrio* spp	+	-	-
44	*Vibrio* spp	+	-	-
45	*Vibrio* spp	-	-	-
46	*Vibrio* spp	+	-	-
47	*Vibrio* spp	+	-	-

Table 6 Mortality of 1 month black tiger shrimp in varies chitosan concentration after 96 hours.

Chitosan (%)	Number of dead shrimp			
	Replication 1	Replication 2	Replication 3	Mortality (%)
0.0	0	0	0	0
0.1	0	0	0	0
1.0	1	0	0	3.3
2.0	5	5	4	46.7

Note: Weight of experimental shrimp is 3.77 ± 0.54 grams.

Table 7 Water quality of the experimental safety test.

Time (Hours)	Parameters	Chitosan (%)			
		0.0	0.1	1.0	2.0
0	Dissolve oxygen (mg/l)	6.32	6.32	6.32	6.32
	pH	7.60	7.60	7.60	7.60
	Temperature (°C)	28.6	28.6	28.6	28.6
	Alkalinity (mg/l)	105	105	105	105
	Ammonia (mg/l)	0.0308	0.0308	0.0308	0.0308
	Salinity (ppt.)	20	20	20	20
48	Dissolve oxygen (mg/l)	6.25	6.11	6.30	6.04
	pH	7.70	7.40	7.60	703
	Temperature(°C)	29.1	29.0	28.9	29.1
	Alkalinity (mg/l)	101	103	105	100
	Ammonia (mg/l)	0.0406	0.0340	0.5011	0.0436
	Salinity (ppt.)	20	20	20	20
96	Dissolve oxygen (mg/l)	6.22	6.00	6.22	6.11
	pH	7.60	7.50	7.20	7.20
	Temperature (°C)	28.7	28.7	28.6	28.8
	Alkalinity (mg/l)	102	103	100	100
	Ammonia (mg/l)	0.040	0.0510	0.0479	0.0471
	Salinity (ppt.)	20	20	20	20

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LITERATURE CITED

A.O.A.C. 1984. **Official methods of analysis**. 14th ed. Association of official Analytical chemistry. Washington, D.C. 1141 p.

Geiss, H.K., H.D. Piotrowski and V. Hingst. 1985. Evalution of API 20 NE in routine diagnostics of nonfermenting gram negative rod shaped bacteria. **Zbl. Bakt. Mikrobiol. Hyg. A-Med**. 259: 35-42.

Karunasagar, I. , R. Pai, G.R. Malathi and I. Karunasagar. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. **Aquaculture** 128: 203-209.

McFarland, J. 1907. The nephelometer: An instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. **J. Amer. Med. Assoc.** 49: 1176.

No, H.K., N.Y. Park, S.H. Lee and S.P. Meyers, 2002. Antibacterial activity of chitosan oligomers with different molecular weights. **Int. J. Food. Microbiol.** 74: 65-72.

Ohtakara, A., M. Izume and M. Mitsutomi. 1988. Action of microbial chitosanases on chitosan with different degrees of deacetylation. **Agricultural and Biological Chemistry** 52: 3181-3182.

Razdan, A. and D. Pettersson. 1994. Effect of chitin and chitosan on nutrient digestibility and plasma lipid concentrations in broiler chickens. **Br. J. Nutr.** 72(2): 277-288.

Roberts, G.A.F. 1992. **Chitin Chemistry**. The Macmillan Press, Basingstoke, Great Britain.