

EFFECT OF POVIDONE IODINE TREATMENT ON BACTERIAL COMMUNITY ASSOCIATED WITH WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) LARVAE

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ABSTRACT

The efficacy of povidone iodine as a surface disinfectant was assessed in white shrimp (*Litopenaeus vannamei*) eggs and larvae. Eggs and nauplii were exposed to povidone iodine 20 ppm for 20 seconds. Culture-based method and Terminal Restriction Fragment Length Polymorphism (T-RFLP) were used to monitor the total number of bacteria and diversity of the bacterial community associated with shrimp eggs and larva at each developmental stage. Povidone iodine reduced the total culturable bacteria, especially *Vibrio*, on eggs and nauplii, as well as the total bacteria in the whole community as estimated by T-RFLP results. Povidone iodine also reduced the diversity of bacterial community and altered the evenness of phylotypes distribution suggesting that the use of povidone iodine as a surface disinfectant in shrimp aquaculture should be reconsidered.

Keywords: bacterial community, povidone iodine, shrimp, T-RFLP

INTRODUCTION

White shrimp (*Litopenaeus vannamei*) is one of the major cultured shrimp species in the world. Its production increased 120% between 2002-2003 and predicted will be increasing more in later years (FAO, 2006). An increasing demand had forced intensive culture of this species, which brought many problems due to increasing disease outbreaks caused by microorganisms that lead to mass mortality. Recently, it has been widely accepted that microbial community plays a major role in aquaculture. Microbiota which live in association with aquatic animals may enhance host growth and survival by producing some digestive enzymes (Seeto *et al.*, 1996; Sugita *et al.*, 1995; Izvekova, 2006), out-competing pathogenic bacteria (Primavera *et al.*, 2000), and supplying essential compounds important for host metabolism (Sugita *et al.*, 1991). It is preferably to maintain this community in balance rather than growing the shrimps under sterile conditions. Rapid growth of the shrimps occurred in unfiltered pond water, which contain organic particle including bacteria (Moss and Pruder, 1995).

Regular disinfection of the eggs and larvae with approved chemical disinfectants is a common

practice in most commercial shrimp hatcheries. The use of drugs and chemicals for disinfectant in aquaculture facilities is regulated by various federal and state agencies. Treatments must be effective, safe, and cost efficient. Some chemicals that commonly used for disinfectant in aquaculture include povidone iodine, formalin, hydrogen peroxide, and glutaraldehyde.

Povidone iodine is an aquaculture drug with low regulatory priority which is widely used in commercial shrimp hatcheries in Indonesia. Many studies have been conducted to determine the effects of treating the eggs/larvae at various stages of development. Iodine treatment could reduce the bacterial load of white shrimp (*Litopenaeus vannamei*) eggs and larvae without affecting the hatching rate of eggs or survival rate of larvae (Cooney *et al.*, 2005). This chemical was also used for disinfecting eggs of other aquaculture species such as longtooth grouper, *Epinephelus bruneus* (Eissa *et al.*, 2007), red porgy (*Pagrus pagrus*) and white sea bream (*Diplodus sargus sargus*) (Katharios *et al.*, 2007).

Most of the works to study the microbial community of shrimps were done based on the culture-based method. This method has limitation since less than 1% of the bacteria has been

successfully cultured in artificial media until present. Only particular groups of bacteria were recovered by this method, i.e. heterotroph aerobic bacteria. The molecular method based on the amplification of 16S rRNA genes was used to solve this problem. This gene is ubiquitous and highly conserved among prokaryotic organisms, which make it useful as a molecular marker for microbial community analysis. One of the techniques that used this approach was the Terminal Restriction Fragment Length Polymorphism Analysis (T-RFLP). T-RFLP provides several advantages over other techniques because it is saving time and cost especially when many samples are to be analyzed. T-RFLP has been suggested more sensitive and has a greater resolution than other fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (Marsh, 1999).

This research was aimed to characterize the bacterial community associated with white shrimp larvae with povidone iodine treatment using culture-based method and T-RFLP. To our knowledge, this is the first microbial community study associated with shrimp larvae employing molecular-based technique. This work will provide more reliable conclusion about the efficacy of this treatment to control bacterial load at shrimp larvae, since the previous study was conducted using culture-based method which covered culturable bacteria only.

MATERIALS AND METHODS

Sample preparation

Samples of eggs, nauplii, zoea, mysis, and post larvae were stages from single cycle production, obtained from the marine research center hatchery, PT. Central Pertiwi Bahari Lampung Indonesia. Eggs from the same broodstock were divided into 2 groups, respectively the iodine treatment and control group. For the iodine treatment group, eggs were bathed in a 20 ppm povidone iodine solution for 20s before being transferred to hatching tanks, about 5 hours after spawning. The same treatment was applied to the nauplii before being transferred to rearing tanks. Samples were taken after the treatment for eggs and nauplii. Samples of other stages were taken just after the larva reach the stages. At the same time, rearing water were also sampled.

DNA extraction

The amount of 0.01 g of egg and larvae sample was washed 3 times with 0.85% sterile NaCl on sterile filter paper to eliminate non associated microorganisms. One hundred ml of rearing water was filtered through a 0.45 µm nylon membrane and this membrane was used for DNA extraction. Bacterial DNA was extracted from each sample and from the nylon membrane using an Ultra Clean Soil DNA Isolation kit (MoBio, California).

PCR Amplification

63f primer that 5' end labeled (5'-(6FAM) CAGGCCTAACACATGCAAGTC-3') and 1387r primer (5'-CCCGGGAACGTATTCACCGC-3') were used to amplify 16S rRNA gene. Reaction mixtures for PCR contained 100 ng DNA, 1x buffer (New England Biolabs, Massachusetts), 200 µM of each dNTP, 1 U Taq DNA Polymerase (NEB, MA), 5 pmol of each primer, in a final volume of 50 µl. DNA amplification was run with a 3 minutes denature step at 94° C; 30 cycles of 1 minute at 94° C, 1 minute at 55° C, 1 minute at 72° C; final annealing step at 72° C for 10 minutes. The PCR product was treated with mung bean nuclease (NEB, MA) to eliminate pseudo T-RF. Then the PCR product was run on 0,8% agarose gel. The DNA band with approximately 1500 bp in size was excised and purified using the Qiaquick Gel Extraction Kit (Qiagen, Germany).

Restriction Enzyme Digestion

Each purified PCR product was digested with 2 enzymes: respectively *Sau3A1* and *RsaI* (NEB, MA) in separate tubes. The reaction mixtures contained 5U enzyme, 1x buffer, 100-200 ng DNA in total volume of 20 µl and incubated at 37°C overnight. Digested DNA was then purified with Qiaquick Nucleotide Removal Kit (Qiagen, Germany) and eluted with 30µl elution buffer.

T-RFLP Analysis

1 µl of digested DNA was mixed with 0.5 µl of HD-400 [ROX] as internal standard, denatured at 95°C for 5 minutes, then placed on ice. The lengths of various T-RFs were analyzed using an ABIprism™ 3100 Automated DNA Sequencer and determined using the GeneScan Programme

(Perkin Elmer, Norwalk). The sizes of T-RFs were compared with the database of Ribosomal Database Project II to identify their closest relatives.

Diversity Analysis

Bacterial phylotype richness (S) was expressed as the total of different T-RF peaks found within each sample. Shannon Wiener index (H') and the evenness (E) was calculated to describe the diversity of community and relative importance of each phylotype within the entire assemblage. H' was calculated as follows: $H' = -\sum (\pi_i) (\ln \pi_i)$ where π_i is the relative abundance of fragments i . Evenness was measured as follow: $E = H' / H_{max}$ where $H_{max} = \ln S$ (Margalef 1958).

Culture and Identification of Isolates

0.01 g of eggs and larva from each stage were homogenated in 0.85% NaCl solution. One ml Serial dilutions were made in 0.85% NaCl solution from the sample homogenate. For each dilution, 0.1 ml aliquot was plated on SWC (Sea Water Complete) medium for total bacterial count and TCBS medium for total *Vibrio* count. Morphologically different colonies on SWC plates were selected and further purified. All isolates were identified based on the sequence of 16S rRNA gene. The sequences were compared for homology to the NCBI database using BLAST.

RESULTS

Total Bacterial Count

Bathing eggs and nauplii for 20 s in 20 ppm povidone iodine reduced the total bacterial count on SWC medium (Fig. 1.). Approximately 98% and 93% of the total viable bacterial counts on treated eggs and nauplii decreased respectively, comparing to control. Total bacterial count for the group of larva treated with povidone iodine during developmental stages remained lower than the control group until the last stage of larvae development.

Povidone iodine treatment was also affecting the diversity of the bacterial community at eggs and larvae, as shown in Figure 2. The same isolates were found either in the control group or treatment group, but the relative abundance of each isolate was changed. Iodine suppressed the growth of *Pseudoalteromonas* sp. 10Xb1, *Paracoccus zeaxanthinifaciens* strain R-1506, *Alteromonas* sp. DG1302, and *Microbacterium* sp. SMB18. This caused the raise in population of other isolates which seemed to be resistant to iodine treatment, i.e. *Alteromonas* sp. SPB-5 and *Exiguobacterium* sp. JL-42. One other isolate, Flavobacteriaceae bacterium P99-3 was not affected by iodine treatment. Its relative abundance was only slightly different between the treatment and control group of eggs/larvae.

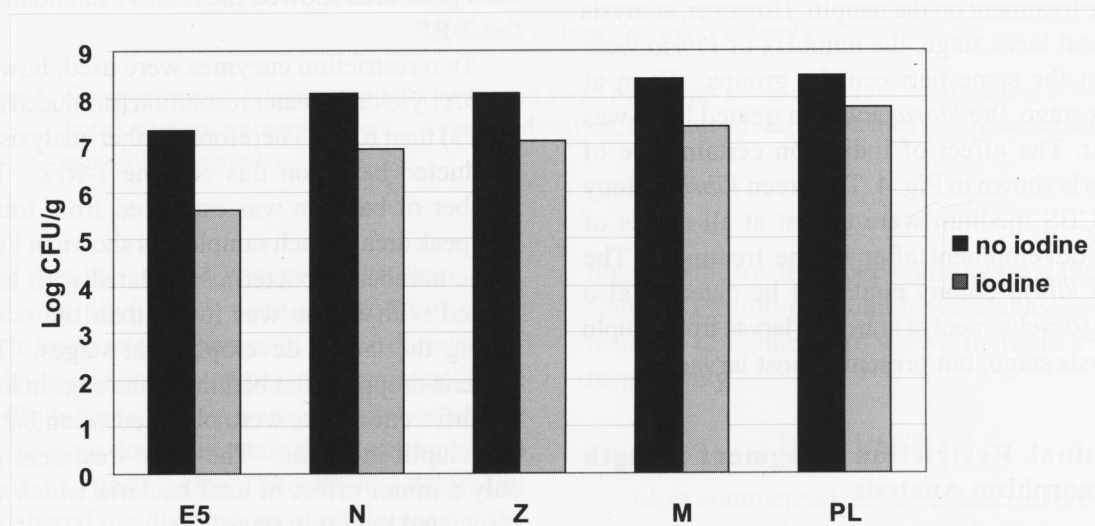


Figure 1. Total bacterial count during shrimp larvae developmental stages after bathed in povidone iodine (20 ppm for 20 seconds). E5=eggs 5 hours after spawning; N=nauplii, Z=zoea, M=mysis, PL=post larvae.

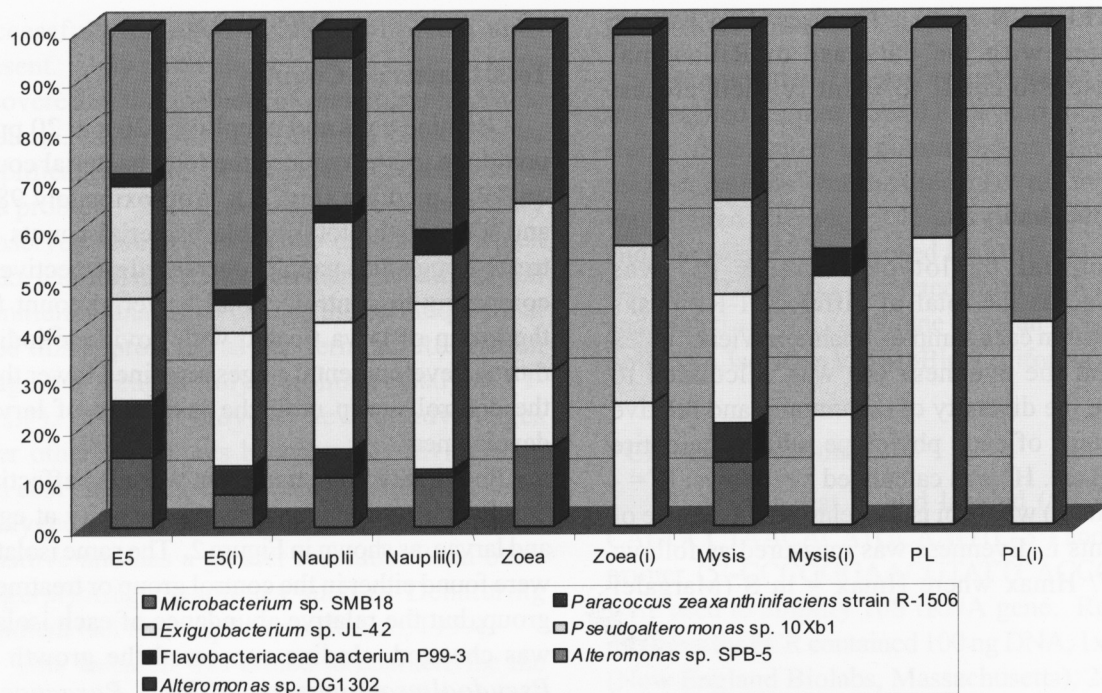


Figure 2. Composition of bacterial community associated with each stage of shrimp larvae developmental stages; E5=eggs 5 hours after spawning; N=nauplii, Z=zoea, M=mysis, PL=post larvae, (i)= bathed in povidone iodine (20 ppm for 20 seconds).

Total Vibrio Count

The effect of povidone iodine treatment on the *Vibrio* group was also examined. Total *Vibrio* count was conducted using TCBS medium. The number of *Vibrio* was lower in the treatment group compared to the control, but only after the bathing treatment of the eggs until the zoea stage (Fig. 3.). No *Vibrio* could be detected at treated eggs. *Vibrio* count reduced approximately 67% after iodine treatment on the nauplii. However, at mysis and post larva stage, the numbers of *Vibrio* were almost the same between the groups. Even at mysis stage, the *Vibrio* count in treated larva was higher. The effect of iodine on certain type of *Vibrio* is shown in Fig. 4. The green *Vibrio* colony on TCBS medium were absent at all stages of larva development after iodine treatment. The green *Vibrio* colony could not be detected also during development of untreated larvae from nauplii to mysis stage, but present at post larvae.

Terminal Restriction Fragment Length Polymorphism Analysis

To see the effect of iodine treatment on the whole bacterial community, molecular-based

technique, Terminal Restriction Fragment Length Polymorphism (T-RFLP) were used. T-RFLP was used to monitor the change in the microbial community of the larvae group treated with iodine comparing to the control, using the same shrimp eggs/larvae sample as the cultured-based method. Sample data consisted of the size in base pair and peak area for each T-RF peak in electrophoregram. One T-RF was considered as a phylotype while each peak area showed the relative abundance of the T-RF.

Two restriction enzymes were used, however *Sau3A1* yielded greater resolution (produced more T-RFs) than *RsaI*. Therefore, further analysis was conducted based on this enzyme T-RFs. Total number of bacteria was estimated from total T-RFs peak area of each sample. As shown in Figure 5, the number of bacteria associated with larvae treated with iodine was lower than the control during the larvae developmental stages. Total bacteria dropped after bathing of the eggs in iodine but different results were obtained when bathing the nauplii in iodine. The same treatment gave only a minor effect in total bacteria which were associated with this stage.

The diversity of the bacterial community associated with the treatment group of larvae was

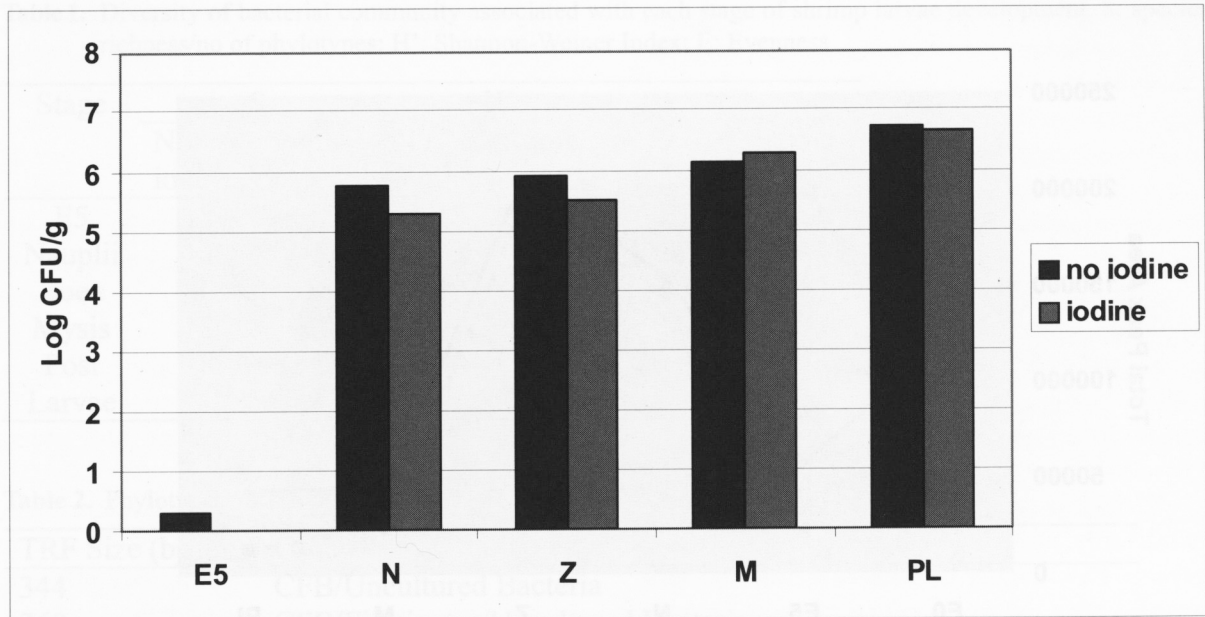


Figure 3. Total *Vibrio* count during shrimp larvae developmental stages after bathed in povidone iodine (20 ppm for 20 seconds). E5=eggs 5 hours after spawning; N=nauplii, Z=zoea, M=mysis, PL=post larvae.

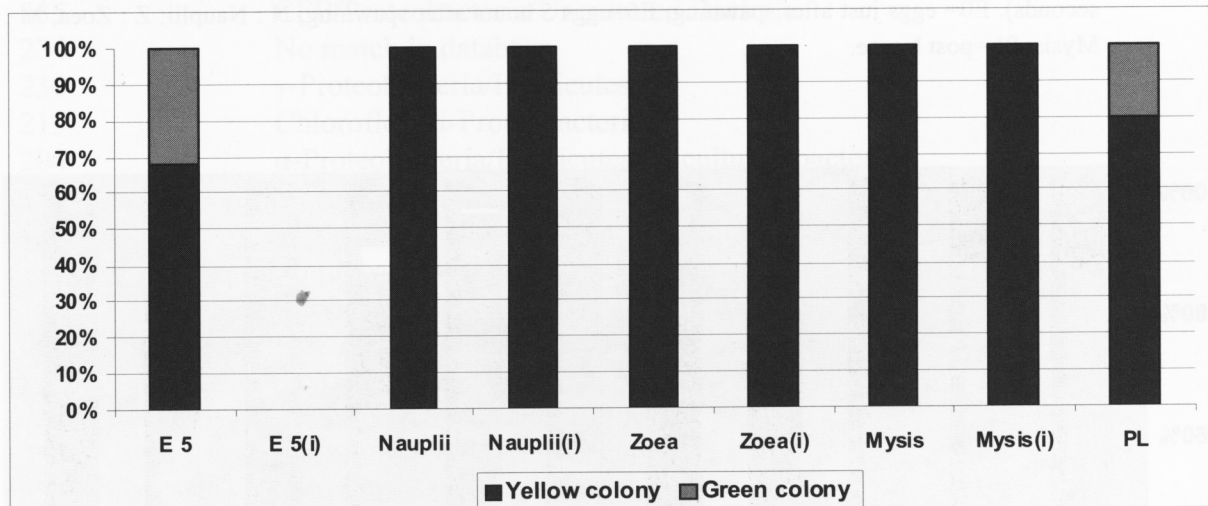


Figure 4. Composition of *Vibrio* associated with each stage of shrimp larvae developmental stages; E5=eggs 5 hours after spawning; N=nauplii, Z=zoea, M=mysis, PL=post larvae, (i)= bathed in povidone iodine (20 ppm for 20 seconds).

lower than the control, except in zoea stage (Fig. 6.). Some phylotypes were affected by iodine which leads to the change in either diversity or distribution of the phylotypes in community (Table 1.). Phylotypes which were resistant to iodine became more dominant while the ones which were sensitive to this treatment became less dominant. This altered the distribution of phylotypes in the community as indicated by the evenness value. At eggs and post larvae, the evenness values were

higher in the treated group while lower at the other stages. Phylotypes which were sensitive and resistant to iodine were shown in Table 2 and 3 respectively.

DISCUSSION

Most commercial shrimp hatcheries preferred to use low regulatory priority drugs to control bacterial content in their products. Povidone iodine is one of the chemical which proved to be safe

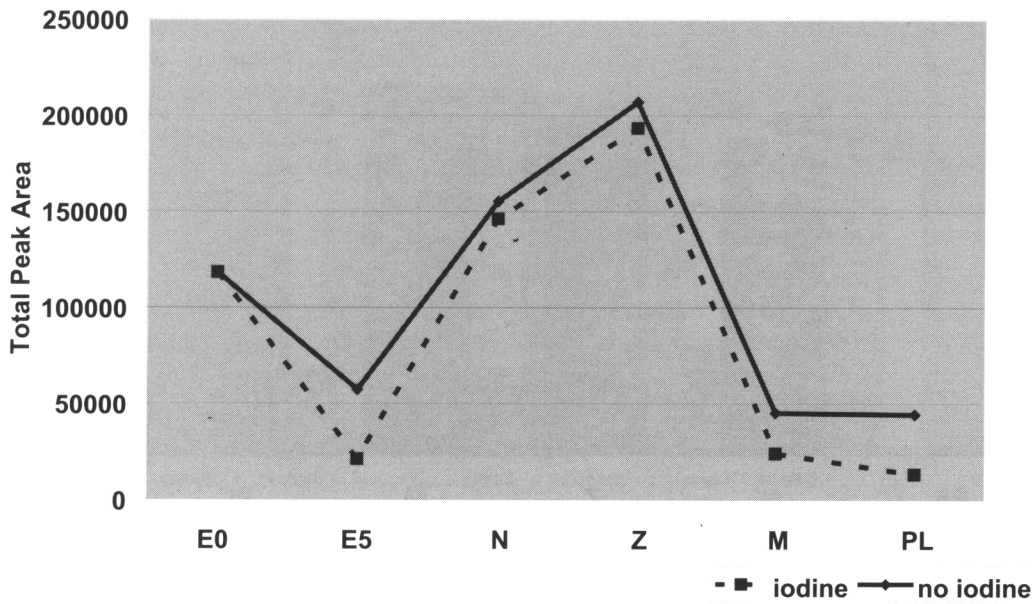


Figure 5. Estimation of total bacteria based on T-RF total peak area after bathed in povidone iodine (20 ppm for 20 seconds). E0= eggs just after spawning; E5=eggs 5 hours after spawning; N : Nauplii; Z : Zoea; M : Mysis; PL=post larvae.

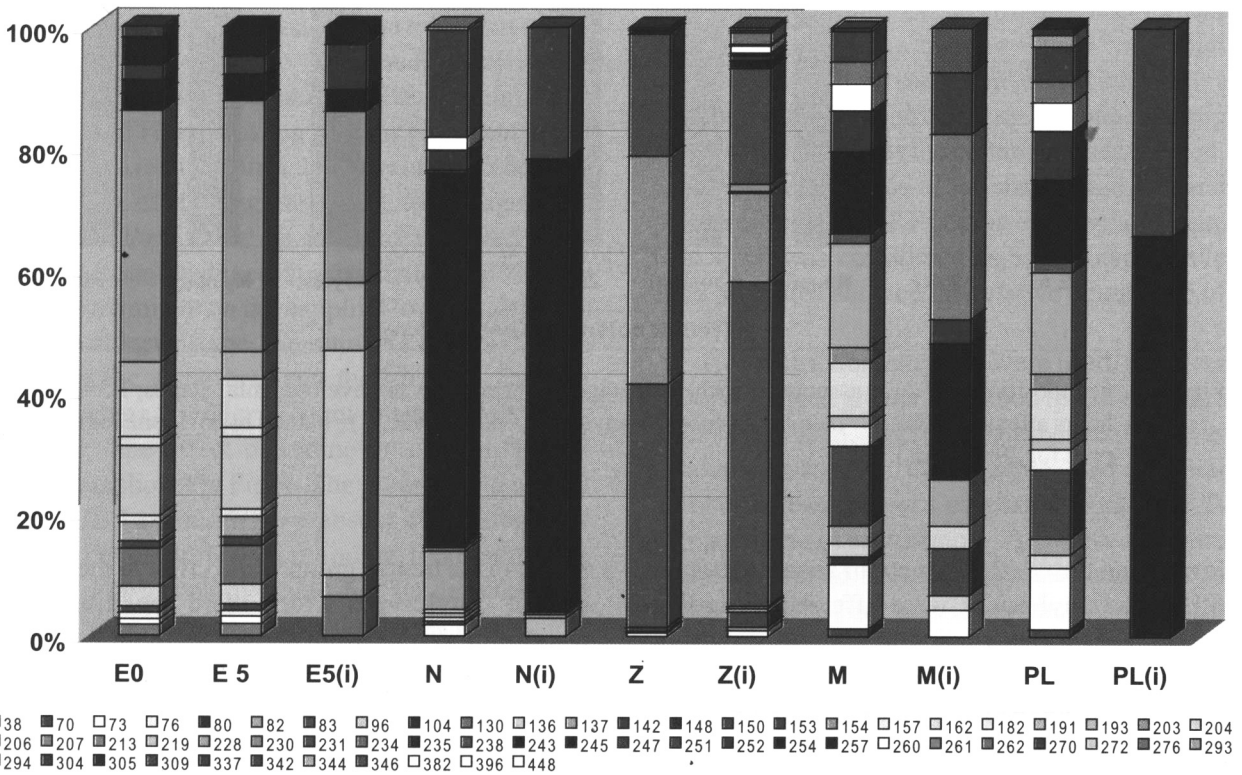


Figure 6. Composition of *Sau3A1* T-RFs during shrimp larvae developmental stages. E0 : eggs just after spawning; E5 : Eggs 5 hours after spawning; N : Nauplii; Z : Zoea; M : Mysis; PL : Post larvae; (i)= bathed in povidone iodine (20 ppm for 20 seconds).

Table 1. Diversity of bacterial community associated with each stage of shrimp larvae development. S: species richness/no of phylotypes; H': Shannon-Weiner Index; E: Evenness

| Stage | S | | H' | | E | |
|-------------|-----------|--------|-----------|--------|-----------|--------|
| | No iodine | iodine | No iodine | iodine | No iodine | iodine |
| E5 | 18 | 8 | 2.18 | 1.68 | 0.75 | 0.81 |
| Nauplii | 14 | 5 | 1.33 | 0.71 | 0.5 | 0.44 |
| Zoea | 8 | 18 | 1.19 | 1.53 | 0.57 | 0.53 |
| Mysis | 18 | 10 | 2.55 | 1.99 | 0.88 | 0.86 |
| Post Larvae | 18 | 2 | 2.58 | 0.64 | 0.89 | 0.93 |

Table 2. Phylotypes Sensitive to Iodine

| TRF Size (bp) | Group |
|---------------|---|
| 344 | CFB/Uncultured Bacteria |
| 260 | CFB/Firmicutes/Uncultured Bacteria |
| 254 | Uncultured bacteria |
| 251 | Firmicutes/Uncultured Bacteria |
| 243 | Uncultured β -Proteobacteria |
| 235 | CFB/Proteobacteria/Uncultured Bacteria |
| 234 | No match in database |
| 230 | γ -Proteobacteria/Firmicutes |
| 219 | Chloroflexi/ α -Proteobacteria |
| 204 | α -Proteobacteria/Firmicutes/Uncultured bacteria |
| 162 | Uncultured α -Proteobacteria/CFB |
| 153 | α -Proteobacteria/ Uncultured bacteria |
| 157 | Proteobacteria/Firmicutes/CFB |
| 150 | Firmicutes/CFB |
| 96 | No match in database |
| 83 | No match in database |
| 76 | Uncultured α -Proteobacteria |
| 73 | Uncultured bacteria (isolated from Pig) |
| 38 | Firmicutes/ Uncultured bacteria |

Table 3. Phylotypes Resistant to Iodine

| TRF Size (bp) | Group |
|---------------|--|
| 270 | Firmicutes/Uncultured bacteria |
| 252 | Firmicutes/uncultured bacteria |
| 245 | CFB/ uncultured bacteria |
| 228 | Proteobacteria |
| 193 | α -Proteobacteria |
| 154 | Uncultured β -Proteobacteria |
| 130 | Bacterium 072822 (isolated from paddy field) |

and effective for this purpose and also approved by various federal and states agencies in importer countries. Povidone iodine is usually used to bath eggs when collecting them for transfer from spawning tanks to hatching tanks. The same treatment was also applied for nauplii when being transferred to rearing tanks at hatcheries. Aim of this treatment is to minimize the bacterial inoculant which could be dangerous for the larval survival. Applying of povidone iodine for eggs could minimize the possibility of vertical transmission of pathogen from infected broodstocks to their progeny (Eissa *et al.*, 2007) since the pathogen is transmitted by egg shell contamination.

Povidone iodine was used for bathing eggs and nauplii only, not continuously added to the larvae culture. The number of total culturable bacteria dropped after the treatment (at eggs and nauplii stages), but slowly recovered at later developmental stages. The recovery of total culturable bacteria count suggesting that povidone iodine effect was temporal. Povidone iodine also affected the diversity of the bacterial community associated with white shrimp larvae. Not all bacteria of the community were sensitive to povidone iodine treatment which changes the composition of community. However, not like its temporary effect on total bacterial count, the effect of iodine that suppressed the growth of certain bacteria lowered diversity at later stages of larvae development.

The short term effect of povidone iodine was also observed for the *Vibrio* group as its effect on total bacterial counts above. According to Wen Yu *et al.* (1994), bacterial population altered from dominant Gram positive to dominant Gram negative when for the first time introduced with *Artemia* nauplii as feed for *P. monodon* larvae at zoea III stage. When *Artemia* cyst was given to the larvae culture, it broke and released its reserved gliserol to rearing water (Sorgeloos *et al.*, 1986). Gliserol is an organic substrate which could be efficiently used by *Vibrio* spp. and this caused proliferation of the *Vibrio* group. Further more, the *Artemia* nauplii could be a vector for *Vibrio* spp to enter the gut of white shrimp larvae making this group dominant in the bacterial community of the larvae. Since iodine only had a short term effect, when it was applied at early stages of larvae development, it might not protect the shrimp larvae against blooming of *Vibrio* spp. due to first feeding of *Artemia* at mysis stage.

Comparing to its effect on total bacteria, iodine seemed to have a lower effect on the *Vibrio* group, as shown by only a slight decrease in the number of *Vibrio* which was detected after iodine bathing treatment for nauplii and a quicker recovery at later stages compared to the total bacteria. These results suggested that most *Vibrio* associated with white shrimp larvae were relatively resistant to povidone iodine. The mechanism by which *Vibrio* resistant to povidone iodine is still unclear, but it might be due to the lipopolysaccharide capsules that surrounding *Vibrio* cell prevent the iodine ion to penetrate into the cell (Pyle and McFeters, 1989). The *Vibrio* also have the ability to form biofilm that may protect the cells from antimicrobial substances (Karunasagar *et al.*, 1996). The green *Vibrio* colony was the one that mostly affected by povidone iodine, but this was not the dominant type of *Vibrio* associated with white shrimp larvae. Therefore, the iodine treatment has a lower effect on the total *Vibrio* count as mentioned above. However, no *Vibrio* could be detected on SWC medium suggesting that this group was not dominant in bacterial community of white shrimp larvae.

Using the T-RFLP technique, the effect of povidone iodine on unculturable bacteria can be monitored as well. The T-RFLP was more sensitive comparing to the culture-based technique. The diversity of bacterial communities was greater using T-RFLP, although T-RFLP had limitation in precise identification of bacterial species. All isolates obtained by culture were not detected by T-RFLP suggesting that mostly dominants bacteria associated with white shrimp larvae were unculturable. This result justified the superiority of T-RFLP over the culture-based method to study the effect of povidone iodine on bacterial community associated with white shrimp larvae.

Povidone iodine decreased the number of total bacteria through entire larval developmental stages. Only a slight decrease in the total bacteria observed after the treatment for nauplii, comparing to eggs. This result suggested that the composition of the bacterial community in eggs and nauplii were different and affected in different ways by iodine treatment. However, at later stages of larvae development, the total bacteria remained lower in the treatment group comparing to control group.

How iodine influenced the bacterial community at a particular stage was depended on the composition of the bacterial community associated

with that stage, since iodine seemed to have different effect on diverse groups of bacteria. Comparing to the bacterial community associated with untreated larva, the profile of the bacterial community in eggs and nauplii after the iodine treatment could give information whether phylotypes were sensitive or resistant to iodine. Most of the phylotypes which were sensitive to iodine belonged to the proteobacteria group, as shown in Table 2. This explains why in eggs the iodine treatment effectively reduced the total number of bacteria while in nauplii there was only a slight decrease in total bacteria after the treatment. In nauplii, the dominant phylotype (254 bp) which belonged to bacteroidetes group was resistant to iodine. However, iodine lowered the diversity of bacterial community both in eggs and nauplii as shown in Table 1. On the contrary, the evenness in eggs microbial community was higher after the treatment, might be because the dominant phylotype in eggs (230 bp) was sensitive to iodine and became less dominant. This would make more even distribution of all phylotypes associated with eggs, which meant no phylotypes were more dominant comparing to the others in the community.

Interesting phenomenon observed at zoea stage. The diversity in the treated group of larvae was higher than in the control group. Even though iodine had only a short term effect, it might have influenced the establishment of the bacterial community associated with later stages of larvae development. The treatment might cause some iodine-resistant phylotypes over growth and possibly persisted in the community associated with later stages after the treatment. On the other hand, at the control group the phylotype which is sensitive to iodine treatment might be survived and dominate the community at later stages of larvae development. At the last stage of larvae development, post larvae, only 2 phylotypes were detected at larvae group treated with iodine, but both had a relative even distribution. However, there was a possibility that the differences of bacterial community composition at zoea, mysis, and post larvae stage in both groups were due to tank-to-tank variations, since treatment group and control were reared at different tanks.

As a conclusion, povidone iodine is quite effective as a surface disinfectant in shrimp aquaculture. Despite its short term effect, iodine could minimize the vertical or horizontal transmission of bacteria to the shrimp larvae and

reduced the possibility of infection of the bacterial inoculum which might be harmful for the larvae survival. Iodine treatment could reduce the bacterial load in eggs or larvae of the shrimp, although not all bacteria associated with shrimp eggs/larvae were sensitive to iodine. Iodine might be a selecting agent for certain groups of bacteria and change the balance in the bacterial community. The negative effect is that useful bacteria might also be suppressed and opportunistic pathogenic bacteria might become dominant. This means that the use of any disinfectant to control the total bacterial load in eggs and larva to increase the shrimp production should be applied with extra caution in order to minimize the problems caused by microorganisms in aquaculture.

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