Philippine Journal of Science
140 (2): 173-178, December 2011
ISSN 0031 - 7683
Date Received: 23 Feb 2011

Moderate Inhibition of Luminous *Vibrio harveyi* by Aqueous Extracts Obtained from the Skin of Tilapia, *Oreochromis* sp.

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The inhibitory effects of aqueous skin extracts obtained from both high saline and freshwater tilapia, *Oreochromis* sp. on luminous *Vibrio harveyi* was conducted using the well-diffusion and co-incubation assays. There was an inhibition of luminous *V. harveyi* until the 10^{-2} dilution of the aqueous extracts from both the freshwater and high saline tilapia strains, with higher frequency of inhibition observed in the latter. In the co-incubation assay, both skin extracts were able to inhibit the number of luminous *V. harveyi*, although the concentration used for the extracts from the high saline tilapia was one-hundred times lower than the extracts of the freshwater strain. These results suggest that the aqueous extracts from the skin of tilapia from different environments could inhibit the growth the luminous *Vibrio* and the effects are more apparent in fish obtained from the high saline rearing conditions.

Key Words: aquaculture, luminous vibriosis, *Oreochromis* sp., skin, tilapia, *Vibrio harveyi*

INTRODUCTION

Luminous *Vibrio* is recognized as an important pathogen of cultured penaeid larvae throughout the Southeast Asian region (Lavilla – Pitogo et al., 1990; Karunasagar et al., 1994). These bacteria are natural inhabitants of the coastal water, and in hatchery systems, eggs may immediately become infected by the bacteria that go with the fecal matter of the spawners.

In the intensive culture of the black tiger shrimp *Penaeus monodon*, virulent strains of *Vibrio harveyi* cause luminous vibriosis that results in devastating mortality of the shrimp larvae in hatcheries (Sunaryanto and Mariam 1986, Lavilla – Pitogo et al. 1990, Karunasagar et al. 1994). Highly virulent strains kill up to almost all of *P. monodon* larvae in baths con taining as little as 10^2 colony forming units (CFU) ml^{-1} (Lavilla – Pitogo et al. 1990) whereas many other strains are virulent at 10^6 CFU ml^{-1} (Pizzutto and Hirst 1995).

Because luminous vibriosis has devastating effects on the shrimp larval systems (Lavilla – Pitogo et al., 1990), it is imperative that measures for their control be developed. Chemical treatment of luminous vibrosis in shrimp larvae is quite limited because of the ineffectiveness of existing and readily available drugs, possible development of resistance in bacteria, human health hazard, high toxicity and the prohibitive cost of these drugs.

Various techniques to prevent outbreaks of luminous vibriosis in shrimp ponds have been introduced. One of the techniques that have been reported to work is the" green water" system (Corre et. al., 2000), wherein beneficial green microalgae are favored to grow in shrimp ponds to control the population of luminous *Vibrios*. Tendencia et al (2005) reported that tilapia present in the water directly inhibit the growth of *V. harveyi*. Two major mechanisms

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are considered for the direct inhibitory effect of tilapia on *V. harveyi*. One is the inherent property of tilapia, such as mucus and other metabolites which could have direct inhibitory action against *V. harveyi*; and the other is the microflora associated with tilapia culture that could have a competitive effect on *V. harveyi*.

There have been several studies on the antibacterial, antifungal and cytotoxic activities of the mucus from different species of fish (Austin and McIntosh, 1988; Magariños et al. 1995; Ebran et al. 2000; Hellio et al. 2002). This is because, mucus, which is associated with the fish skin, contains defense factors specific and nonspecific antimicrobial compounds including complement factors, lysozymes, proteases, C–reactive protein, immunoglobulin, lectin–like molecules, agglutinins and glycoproteins (Alexander and Ingram 1992; Ellis 2001; Magnadóttir 2006). Aside from the mucus, the skin also expresses some genes related to immunity including antimicrobial peptides, cytokines, complements, major histocompatibility complex (MHC) and immunoglobulins (Lindenstrøm et al. 2003; Sigh et al. 2004; Gonzalez et al. 2007; Forlenza et al. 2008). These genes that are located in the skin produce substances which are then released to the surface and integrate with the mucus, thereby enhancing the first line of defense in fish against pathogens.

It is a common practice among shrimp famers to stock tilapia together with shrimp in order to lower the incidence of luminous vibriosis during the grow-out phase (Cruz et al. 2008). Despite of the number studies done on the beneficial effects of co-culturing tilapia with shrimp in ponds (Leaño et al. 2005; Tendencia and de la Peña 2003; Tendencia et al. 2004, 2005, 2006) as well as the investigation of epidermal mucus of tilapia and their effects on luminous *Vibrio* (Lio-Po et al. 2005), there have been no studies done to determine the presence of antimicrobial factors in the skin of tilapia that effectively inhibits the growth of the pathogenic bacteria. Hence, this study was conducted to test for the presence of antagonistic activities against luminous *V. harveyi* from the aqueous skin extracts of both high saline and freshwater tilapias.

MATERIALS AND METHODS

Experimental fish and preparation of skin extract

High saline tilapias (*Oreochromis mossambicus*) approximately 40-50 g in weight were procured from the Brackishwater Aquaculture Center of the University of the Philippines in the Visayas, whereas the freshwater strains (*Oreochromis* sp. GIFT strain) approximately 50-60 g in weight were obtained from the Freshwater Aquaculture Center of the same institution. Both strains were of the same age. Individual fish was placed in a bucket with water containing 2-phenoxyethanol (150 ppm) as anaesthetic. Once the fish was fully anaesthetized, it was killed by a strong blow on the head. The scales were aseptically removed and the skin was excised using a sharp blade. Extreme care was taken in order to prevent contamination of the skin with blood and underlying muscle tissues. The skin was weighed, added with an equal amount of normal saline solution (w/v) and homogenized using a tissue grinder. This was followed by centrifugation at 800 rpm at 4°C for 10 minutes. The supernatant was removed, passed through a 100 µm filter, adjusted to a protein concentration of 50 mg ml^{-1}, aliquoted in 1 ml stock and kept at -20°C until use. Five fish (75-100 g) from each rearing condition were used in this study.

Source of *Vibrio harveyi*

The pathogen, *Vibrio harveyi* (0728-26 isolate) was obtained from a previous study (Huervana et al., 2006). This isolate has been tested to cause luminous vibriosis in shrimp. A single colony of the bacterium was inoculated in Nutrient broth supplemented with 1.5% sodium chloride (NaCl) and cultured overnight at room temperature with mild shaking. The density of the bacteria was spectrophotometrically determined at OD600 and the initial density was 6.05 x 10^6 colony forming units per milliliter (cfu ml^{-1}).

Anti-bacterial assay

The reproducible antagonistic activity of the skin extracts against *V. harveyi* was tested using a well-diffusion assay (Ravi et al., 2007). Briefly, 100 µl of the bacterium at a concentration of 10^3 CFU ml^{-1} was plated on Nutrient agar supplemented with 1.5 NaCl. After 1 h, wells with a diameter of 6 mm were made and filled with 25 µl of the serially diluted (ten-fold dilutions) skin extracts. Wells added with normal saline solution served as the control for the experiment. The plates were incubated at 28°C and observed for the zone of inhibition after 24-30 h. The zone of inhibition on the agar plate was scored following Ravi et al., (2007).

Co-incubation assay

In the co-incubation assay, the growth of the luminous bacteria when incubated in the skin extracts was assessed. Similar volumes (100 µl) of the skin extracts from both the freshwater (original concentration) and the high saline (10^{-2} dilution) strains were added with an overnight culture of luminous bacteria at a concentration of 10^3 CFU ml^{-1} and thoroughly mixed. Normal saline solution incubated with the luminous bacteria served as the control. The skin extract-bacteria mixture and the control were allowed to stand at 28°C for 1 h and 100 µl of the mixture was plated onto Nutrient agar with 1.5% NaCl. Luminous bacteria were counted in dark room for observation of

luminescence after 24-30 h.

Statistics

The presence or absence (in terms of percentage) of the zones of inhibition on the agar plates was determined. Chi square (χ^2) test was used to determine the significant differences in the antagonistic activity among the different dilutions with the control. The number of colonies of the luminous bacteria on the Nutrient agar was transformed to their $\log_{10}$ values and expressed as means$\pm$SD. Student's *t*-test was used to determine differences between the colony forming units of luminous bacteria that were co-incubated with the skin extracts and the control. Computations were done using a statistical software (Systat ver. 8, Chicago, Il, USA) and all probability values were set at the 0.05 level of significance.

RESULTS

The results of the antibacterial assay using aqueous skin extracts from high saline tilapia are shown in Table 1. Zones of inhibition in bacterial growth were observed in 5 out of 5 replicates (100%), 4 out of 5 replicates (80%) and 3 out of 5 replicates (60%) at the 10^0, 10^{-1}, and 10^{-2} dilution, respectively. At a dilution of 10^0, the most of the replicates (3 out of 5 replicates) had zones of bacterial inhibition of at least 6 mm. On the other hand, at 10^{-2} dilution the zone of bacterial inhibition was < 3mm in 3 out of 5 replicates. There was no inhibition of the luminous bacteria at the 10^{-3} through the 10^{-5} dilution

Table 2. Antagonistic activity against luminous Vibrio harveyi of aqueous skin extracts from freshwater tilapia.

	Dilution						
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Control
Repliate 1	-	-	-	-	-	-	-
Repliate 2	++	+	-	-	-	-	-
Repliate 3	+	-	-	-	-	-	-
Repliate 4	-	-	-	-	-	-	-
Repliate 5	++	+	+	-	-	-	-

Legend: - absence of inhibition; + inhibition zone < 3mm; ++ inhibition zone >3mm but <6mm; +++ inhibition zone >6mm (Ravi et al. 2003)

as well as in the control group. Further analysis showed that the inhibition of luminous bacteria at the 10^{-2} dilution was significantly different from the control. In the co-incubation assay using the 10^{-2} dilution of the skin extract with luminous bacteria, there was significant reduction in the total count of luminous bacteria in comparison with the control (Figure 1a).

The antagonistic activity of the skin extracts from the freshwater strain showed zones of inhibition of the bacterium in 3 out of 5 replicates (60%), 2 out of 5 replicates (40%) and 1 out 5 replicates (20%) of the luminous bacteria at the 10^0, 10^{-1}, and 10^{-2} dilution, respectively (Table 2). At 10^0 dilution, the zone of bacterial inhibition was between 3 mm and 6 mm in two replicates. No antagonistic activity was observed at higher dilutions (starting at 10^{-3}) of the skin extract. Significant antagonistic activity from the control was only observed at the 10^0 dilution. Using the 10^0 dilution of the skin extract for the co-incubation assay, there was significant reduction in the total count of luminous bacteria than the control group (Figure 1b).

Table 1. Antagonistic activity against luminous Vibrio harveyi of aqueous skin extracts from high saline tilapia.

	Dilution						
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Control
Repliate 1	++	+	-	-	-	-	-
Repliate 2	++	+	+	-	-	-	-
Repliate 3	+	+	+	-	-	-	-
Repliate 4	+	-	-	-	-	-	-
Repliate 5	++	++	+	-	-	-	-

Legend: - absence of inhibition; + inhibition zone < 3mm; ++ inhibition zone >3mm but <6mm; +++ inhibition zone >6mm (Ravi et al. 2003)

DISCUSSION

In the present study, we compared the aqueous extracts obtained from the skin of both freshwater and high-saline tilapias on their ability to inhibit the growth of pathogenic luminous *Vibrio harveyi* in laboratory conditions. We demonstrated that the aqueous skin extracts obtained from the skin of the high-saline tilapia strains had better antagonistic activity against luminous *V. harveyi* in comparison with aqueous extracts from the freshwater strain.

Tests done with the epidermal mucus of the jewel tilapia, *Tilapia hornorum* that was co–cultured with tiger shrimp, *P. monodon* in a "green water" culture system showed that luminous *Vibrio* is not part of the resident microbiota of the fish mucus (Tendencia et al., 2004). In addition, the tilapia mucus effectively eliminated the luminous *Vibrio* in less than 3 hours after exposure to the mucus suspension. Later it was demonstrated that the mucus obtained from jewel tilapia has an inhibitory effect on some bacteria, and believed to contain certain anti–*V. harveyi* factors (Lio – Po et al. 2005). However, the preceding studies have explored on the bactericidal activities of mucus, and that no studies were conducted on the antibacterial properties exhibited by the skin of tilapia on luminous *Vibrios*. Recently it has been shown that extracts from the

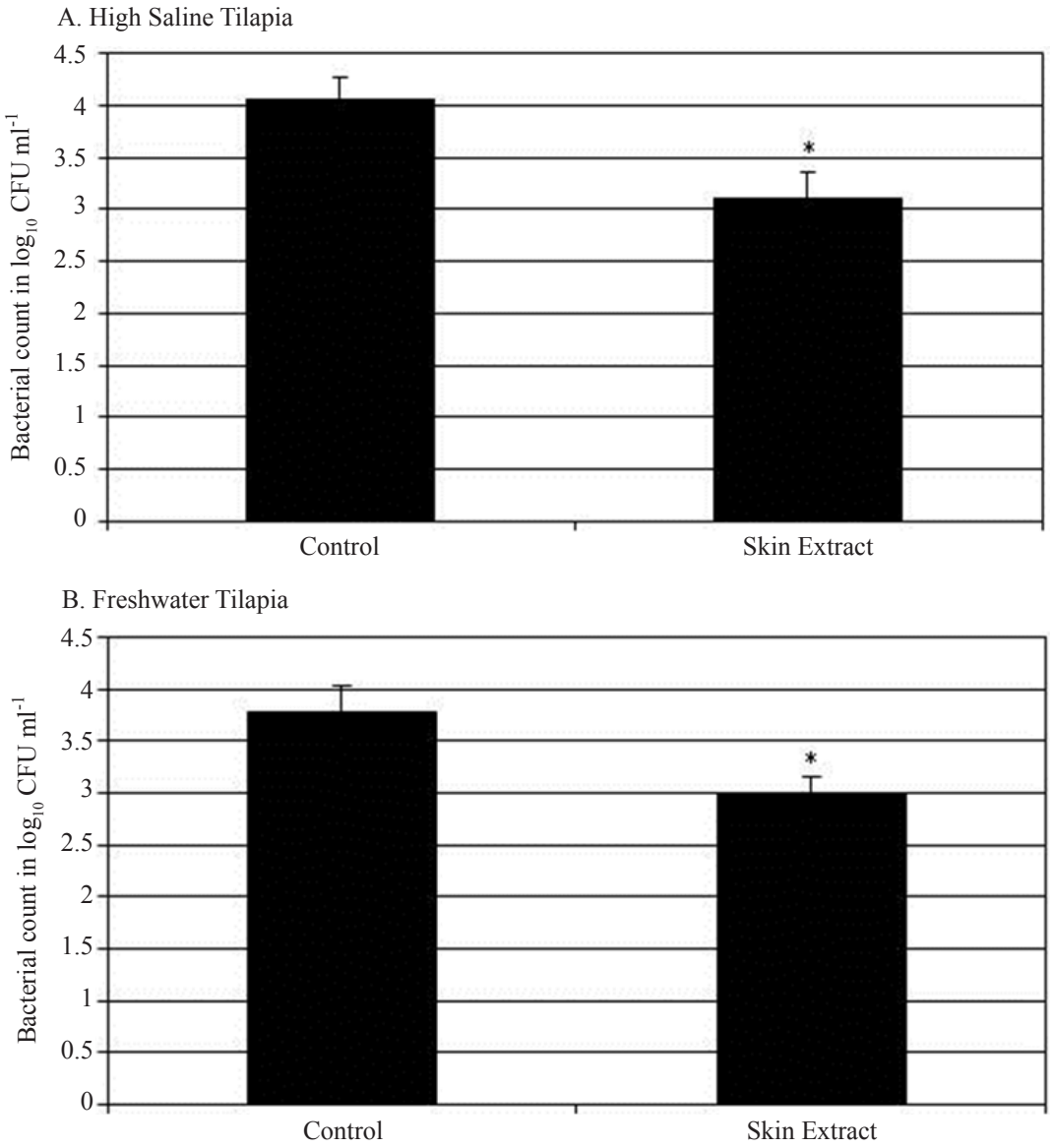

Figure 1. Inhibition of luminous *V. harveyi* during co-incubation with aqueous skin extracts from (A) high saline (10^{-2} dilution) and (B) freshwater tilapia (10° dilution). Column bar with asterisk indicates significant difference at $p < 0.05$. n=5.

various tissues and organs, including the skin of a cold-water fish, *Gadus morhua* exhibited potent antimicrobial activity against different bacterial pathogens (Ruangsri et al. 2010). This suggests that in addition to epidermal mucus, there are certain molecules present in the fish skin with antimicrobial properties. This was observed in *G. morhua* in which the skin has moderate to high expression levels of immune-related genes, which would eventually produce compounds that are released together with the mucus (Caipang et al. 2011). Such conditions may also be true for tilapia, which is a tropical species, and the identification of these immune-related genes from this fish warrants further studies. From a tropical perspective, our study showed that aqueous extracts from the skin of both the high saline and freshwater tilapia also possessed antimicrobial factors that are antagonistic to luminous *Vibrio*. From an aquaculture point of view, it is a common practice that high saline tilapias are being cultured together with shrimp in ponds because of the perceived beneficial effects of the former in controlling luminous vibriosis (Cruz et al. 2008). Here, we have provided evidence that the skin and their secreted products obtained from high saline tilapia have anti-luminous *Vibrio* factors. In comparison, with the skin extracts from freshwater tilapia, the extracts obtained from the high saline strain had apparently better antagonistic activity against luminous *Vibrio*. We cannot explain the underlying factors and/or mechanisms that account for such differences, although

an earlier study on the responses of leukocytes between an infected and non-infected tilapia showed differences in their phagocytic ability (Belotsky et al. 1998). It is possible that the rearing environment, the strain of the fish, size and age as well as the different exogenous factors that the fish are exposed to, could contribute to such differences in their subsequent response in the mucus. Future studies shall focus on identifying and characterizing the different antimicrobial factors from the skin of tilapia, testing skin extracts obtained from using organic compounds such as ethanol or methanol, and elucidating the mechanisms involved in the inhibition on the growth of luminous *Vibrio harveyi*.

In conclusion, the present study has shown that aqueous extract obtained from the skin of tilapia exhibited moderate antibacterial activity against luminous vibriosis, with apparently better antagonistic activity observed from extracts of the high saline strain than the freshwater counterpart. Our results provide evidence on the use of high saline tilapias in the culture of shrimp and lend support on the practice of co-culturing tilapias with shrimp by most aquaculturists in tropical countries as a strategy to curb the growth of luminous *Vibrio* sp. It also showed that aside from the mucus, the skin also contains substances that have inhibitory activity against bacteria. Further studies are recommended to use other extraction agents particularly organic solvents and test whether the extracted substances have antibacterial properties as well as to identify the different substances in the skin mucus of tilapia that have antibacterial activity.

ACKNOWLEDGEMENTS

We are grateful to the National Institute of Molecular Biology and Biotechnology of the University of the Philippines Visayas for allowing us to use the facilities and to Mary Paz Aguana for the technical assistance.

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