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ISOLATION OF THE BACTERIUM, *VIBRIO HARVEYI* FROM CULTURED SHRIMP, *PENAEUS MONODON* AND PRODUCTION OF VACCINES AGAINST THE BACTERIUM

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Abstract: Disease outbreaks detected by the presence of a large number of luminescent post larvae in larval rearing tanks followed by heavy mortalities, have been recorded in most of the shrimp hatcheries in Sri Lanka. Broad spectrum antibiotics, probiotics, and occasionally, imported vaccines have been used indiscriminately to control the disease. In this study, a pathogenic bacterium causing luminescence and heavy mortalities in post larvae was isolated from seven shrimp hatcheries situated in the North Western Province. The bacterium was characterized and identified as a strain of *Vibrio harveyi* using standard microbiological tests and biochemical reactions of bacteria. Two vaccines (Formalin killed and heat killed bacteria) were prepared from the isolated strain of *V. harveyi* and two groups of eighteen day old healthy post larvae (Pl_{18}) were vaccinated separately by immersion technique. Efficacy of both vaccines were then evaluated by challenging vaccinated post larvae after different week intervals from vaccination with the same bacteria. Mean percentage survival of post larvae vaccinated with formalin killed vaccine and heat killed vaccine after 3 weeks from vaccination were 81.62% and 80.22 % respectively which were significantly higher ($p < 0.05$) than that of the post larvae which were not vaccinated and used as the control (35.21 % and 37.89 %) confirming that the vaccines were effective.

Key words: Cultured shrimp, *Penaeus monodon*, Sri Lanka, vaccines, *Vibrio harveyi*.

INTRODUCTION

The number of diseases affecting cultured penaeid shrimp has increased steadily with the expansion and intensification of large scale commercial culture systems.[1, 2] Among the recognized causative agents of infectious diseases of shrimp are viruses, bacteria, rickettsia, fungi, and protozoa. There are heavy production losses due to viral and bacterial diseases.[2]

Bacterial diseases have continued to parallel the growth of the industry in importance.[2] Among the bacterial infections, vibriosis caused by different species of *Vibrio* is recognized to be the most important in initiating mass mortalities in cultured shrimp in East and South East Asia.[3-6] It has been pointed out that there has been wide spread use of antibiotics as a remedial measure against bacterial diseases in shrimp hatcheries. However, it is becoming less and less effective as new strains of bacterial pathogens evolve, which are resistant to commonly used antibiotics. Antibiotics could leave residues in shrimp which could have implications in human health.[7] Therefore, other forms of prevention and control methods are suggested for bacterial diseases and use of vaccines is among them.[8] Antibiotics such as oxytetraxycline, oxolenic acid, and even chloramphenicol are widely used in shrimp hatcheries which could leave residues in shrimp and could be toxic to humans and also develop antibiotic resistance in bacteria.[9]

Shrimp industry in Sri Lanka has experienced two major viral diseases; outbreaks of Monodon Baculo Virus (MBV) disease in 1989/ 90 killing post larvae in hatcheries and White Spot Syndrome Virus (WSSV) disease in 1996 causing heavy mortality in shrimp in grow-out ponds.[10] Recurrence of WSSV disease has been the major cause of low cultured shrimp production in Sri Lanka since 1999.[11] Though there is no published literature, disease outbreaks detected by the presence of a large number of luminescent post larvae in larval rearing tanks followed by heavy mortalities have been recorded in most of the shrimp hatcheries. The luminescence disease condition is not uncommon even in juvenile shrimp in grow-out ponds.

Vaccines composed of inactivated *Vibrio* species [12, 13] are reported to protect different species of shrimp from vibriosis for varying

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periods of time and to improve survival, growth and quality of vaccinated shrimp.[13-16] Possible effects of these vaccines on the defence system of shrimp have been discussed.[17-20] Most of the local hatcheries use broad spectrum antibiotics and some probiotics to control mortality in larval stages while probiotics are widely used in grow-out ponds. Some local shrimp hatchery owners have used imported vaccines to control mortality caused by luminescence disease without positive results. [Personal communication, Mr. Hasitha Thammannagama, Aqua Breed (Pvt.) Ltd, Ambakandawila]. As the strains of bacteria present in local shrimp hatchery systems could be different from the strains present in other countries, it is important to produce vaccines locally rather than importing them. The present study was planned to isolate and identify a common luminous bacterium which caused heavy mortalities of post larvae in some commercial hatcheries, produce vaccines and evaluate the efficacy of the vaccines produced in protecting post larvae when challenged with the same species of bacteria in the live form.

METHODS AND MATERIALS

Isolation and identification of pathogenic luminous bacteria: Seven shrimp hatcheries in the North Western Province, where mortality of post larvae was reported due to luminescence disease condition from May 2002 to January 2003 were selected for the study. The methodology used by Hung-Hung *et al*[21] was slightly modified in isolating the bacterium. Moribund post larvae from each hatchery were randomly selected, and luminous bacterium was isolated on Thiosulphate-Citrate –Bile-Salt Sucrose (TCBS) agar. Stock cultures were prepared and stored separately. Each isolate was identified separately using standard tests [22,23] and the results of biochemical reactions including the tests incorporated to API 20 E miniaturized identification test strip (bio Meriux, France). Morphological and physiological characteristics and biochemical reactions of the bacterial isolates were compared with those of luminous *Vibrio* species isolated from Vietnam and *V. harveyi* type strain from Aquatic Animal Health Resource Institute (AAHRI), Thailand.[23]

Preparation of vaccines: Preliminary investigations were carried out to determine the minimum concentration of formalin and minimum temperature required to kill the isolated bacterium completely. Using stock cultures, confluent growth of the isolated bacterium was obtained and two bacterial suspensions were prepared in sterilized distilled water with 2 % NaCl. Formalin (0.2%) was added to one suspension and the other suspension was heated in a water bath (up to75 ^{0}C) in order to kill the bacteria completely. After confirming that there were no viable bacteria, optical density of the suspension was measured at 350 nm in a spectrophotometer (LED sp -380 Digital, Barnstead and Thermoline, USA) and cell density of both suspensions were made equal by aseptical dilution. The suspensions with bacteria killed by formalin and heat were taken as vaccine 1 and 2 respectively (vaccines were freshly prepared when trials of vaccination were conducted).

Vaccination of post larvae: A static experimental system consisting of 2 L conical flasks was employed for vaccination. Each conical flask was filled with UV sterilized sea water with salinity of 32 gL^{-1} and aerated individually. The water temperature was recorded and hundred apparently healthy, acclimatized, 18 d old post larvae (Pl_{18}) were placed in each conical flask. Vaccination was carried out by immersing the first two groups of post larvae separately in vaccine 1 and vaccine 2 respectively for 5 min. The third group (control) did not receive any vaccination. Vaccinated and non vaccinated post larvae were fed with a commercially available post larval feed (at the feeding regime recommended by the feed manufacturer) and maintained within optimum water quality parameters for periods of 1 wk, 2 wks and 3 wks (three replicates were arranged for each vaccine and for each time period with three controls for each treatment) and subjected to challenge experiments at the end of each period of time separately.

Challenge test: Suspensions of live isolated bacteria (cell density of 5 x 10^4 cfu / mL) were prepared in 0.8% sodium chloride and the post larvae in each test and the control group were

Table 1: Characteristics of isolated bacteria in the present study compared to *Vibrio harveyi* isolated in Vietnam and Thailand.

Characterization test	Bacteria isolated during the present study	*V. harveyi* isolated in Vietnam	*V. harveyi* type strain (43516) from AAHRI, Thailand
Luminescence of bacteria	+	-	-
Gram staining	-	-	-
Haemolysis	+	+	+
Swarming	-	-	-
Motility	+	+	+
Catalase	+	+	+
Oxidase	+	+	+
Pigment production	-	-	-
Growth on TCBS agar	+	+	+
Growth in 0% NaCl	-	-	-
3% NaCl	+	+	+
6% NaCl	+	+	+
10% NaCl	-	-	-
Growth at $4C^0$ - 5 C^0	-	-	-
26 C^0- 27 C^0	+	+	+
ONPG	-	Not done	Not done
ADH	-	-	-
LDC	+	+	+
ODC	+	+	+
CIT	-	-	-
H_2S	-	Not done	Not done
Urease	+	+	+
TDA	-	Not done	Not done
IND	+	+	+
VP	-	-	-
GEL	+	+	+
Utilization of carbon sugars			
Glucose	+	+	+
Arabinose	-	-	+
Mannitol	+	+	+
Sucrose	-	+	+
Inositol	-	Not done	Not done
Sorbitol	-	Not done	Not done
Rhamnose	-	Not done	Not done
Melbiose	-	Not done	Not done
Amygdelin	+	Not done	Not done
Nitrate reduction test	+	+	+
OF-F test	+	+	+
OF-O test	+	+	+

* Characteristics of *V. harveyi* in Vietnam and AAHRI (Thailand) were obtained from published literature.[23]

Table 2: Percentage survival of vaccinated *Penaeus monodon* post larvae (with formalin killed bacteria, vaccine 1 and heat killed bacteria, vaccine 2) after 1 week, 2 weeks and 3 weeks from vaccination.

Treatment	Mean percentage survival ± SE		
	Time from vaccination		
	One week	Two weeks	Three weeks
Formalin killed bacteria (Vaccine 1)	86.51[a] ± 2.441	83.23[a] ± 1.921	81.62[a] ± 3.103
Control	66.04[b] ± 4.625	54.47[b] ± 2.033	35.21[b] ± 4.228
Heat killed bacteria (Vaccine 2)	85.37[a] ± 3.718	82.29[a] ± 1.210	80.22[a] ± 2.996
Control	62.75[b] ± 2.405	51.38[b] ± 3.776	37.89[b] ± 5.001

Values in the same column with different superscript are significantly different from each other ($p<0.05$; One way ANOVA and Tukey's pairwise test)

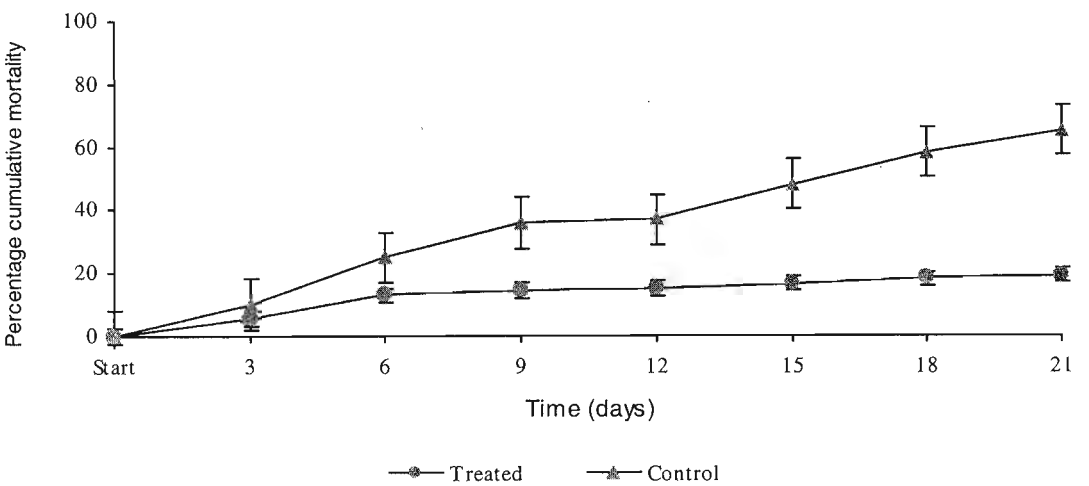

Figure 1: Mean percentage cumulative mortality of *Penaeus monodon* post larvae (±S.E.) treated with formalin killed isolated bacteria (vaccine1) when challenged with the same bacteria in live form.

challenged separately by immersing in the suspension for 5 min. After the challenge experiment, mortality of juveniles in each group was recorded daily for 3 wks and randomly selected moribund shrimp juveniles were used to inoculate TCBS agar plates separately to investigate whether they were infected with the challenged bacteria.

RESULTS

Results of the standard microbiological tests and biochemical reactions of isolated bacteria, including the tests incorporated to API 20E rapid diagnostic system performed to identify the bacteria are given in Table 1 together with the characteristics of *V. harveyi* isolated in Vietnam and Thailand.

It was found that the bacteria isolated from all 7 hatcheries had the same characteristics and were almost identical to *V. harveyi* isolated in Vietnam and Thailand (Table 1); therefore the bacteria isolated could be a strain of *V. harveyi*.

Mean percentage survival recorded for vaccinated post larvae after 1 week, 2 weeks and 3 weeks from challenging with live bacteria was significantly higher than that of non vaccinated post larvae ($p< 0.05$; Table 2). This indicates that vaccinated eighteen day old post larvae (with each vaccine) have achieved protection against the tested strain of *V. harveyi* for a period of 3 weeks while non vaccinated group was susceptible to the luminous vibriosis caused by the same strain of *V. harveyi*. Figures 1 and 2 show the mean cumulative mortality (±S.E.) of vaccinated and non vaccinated post larvae over 3 weeks.

Moribund juveniles from both control and experimental groups that were inoculated on TCBS agar resulted in bluish green colonies with luminescence in the dark confirming that they were infected with *V. harveyi* used for the challenged experiment.

DISCUSSION

Gram negative bacteria of the genus *Vibrio* have frequently been implicated worldwide in cultured penaeid shrimp disease outbreaks in all phases of production.[5,6] The major biochemical characteristics of luminous bacteria isolated from seven hatcheries during the present study were similar to those of *V. harveyi* strains isolated from a disease outbreak in a shrimp hatchery in Vietnam and those of the typed strain (43516) from the AAHRI, Thailand. Therefore, it is concluded that the isolated luminous bacterium in this study is a strain of *V. harveyi*.

Both vaccines prepared with the isolated strain of *V. harveyi* (formalin killed bacteria and heat killed bacteria) protected post larvae of *P. monodon* from the bacterium, *V. harveyi* in live form (when exposed after 1 week, 2 weeks and 3 weeks from vaccination) resulting higher survival (above 80%) compared to post larvae that were not vaccinated (less than 38%). Most of the vaccines available in the market are simple bacterins which have been chemically or heat attenuated to alter the virulancy.[12-15] The vaccines produced in this study is similar in this respect.

A vaccine produced with formalin inactivated strains of *V. harveyi, V. alginolyticus, V. parahaemolyticus* and *V. vulnificus* has improved disease resistance, given protection against vibriosis and luminous vibriosis (resulting higher survival).[13] The growth rate and quality of vaccinated shrimp were also significantly improved in the hatchery and in the grow out stage.[13,16]

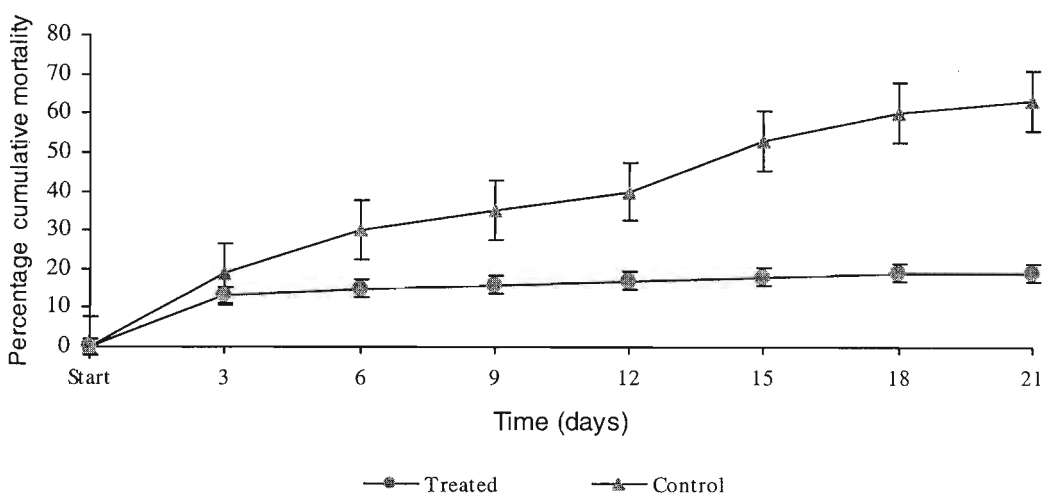

Figure 2: Mean percentage cumulative mortality of *Penaeus monodon* post larvae (±S.E.) treated with heat killed isolated bacteria (vaccine 2) when challenged with the same bacteria in live form.

Increased survival rates observed in shrimp post larvae during the present study after vaccination with both vaccine preparations could have been due to a combination of immunological reactions by the post larvae in response to the attenuated bacteria. Various aspects of *Vibrio* vaccines have been studied; heat killed *Vibrio* cells enhanced the phenoloxidase activity (in the proPo-activating system[17])as immunostimulants.[15] Higher survival rate and higher phagocytic index has been observed in *vibrio* vaccinated shrimp when compared to shrimp that were not vaccinated.[3] Oral, bath and injection administration of immunostimulants , such as *Vibrio* bacterin, β-glucan and peptidoglycan could increase the resistance of shrimp against microbial infections.[15] β- glucan and peptidoglycan are bacterial cell wall components.[18] These components in the cell walls of dead *Vibrio* cells in formalin killed and heat killed vaccines in this study could have acted as immunostimulants resulting in higher survival during the challenge experiment. It has been pointed out that the non-specific immune system of shrimp functions as an immunological memory to prevent new infections caused by pathogens.[19] Following immersion or oral ingestion of *Vibrio* bacterin, the shrimp showed significant resistance against the challenge with *V. harveyi*, a virulent bacterium.[3] The study showed that, under laboratory conditions post larvae of *Penaeus monodon* when exposed to formalin and heat killed *V. harveyi* strain could achieve an effective protection against luminous vibriosis caused by the same bacteria up to 3 weeks indicating potential use of these vaccines.

Some researchers have pointed out that as shrimps lack a well developed specific defence system, any enhancement of its defences against pathogens can only be for a short time.[20] Others have showed that the immunity achieved by post larvae of *Penaeus sytlyrostris* after vaccination with a *Vibrio* species lasted for 4 months which is sufficient to complete one culture cycle.[15]

Results of this study do not support the view that vaccination of shrimp is meaningless, since they do not have immunological memory response.[8] It has also been stated that increasing resistance of shrimp to a single specific pathogen cannot protect them from other pathogens during the culture period.[15] Therefore, in order to protect shrimp from vibriosis, it is important to isolate and identify other pathogenic *Vibrio* species common in shrimp hatcheries and grow out systems in Sri Lanka and develop vaccines incorporating all these *Vibrio* species.

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