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VIRULENCE DETERMINATION AMONG *VIBRIO HARVEYI HATCHERY* ISOLATES THROUGH HAEMOLYSIS AND GROWTH CONSTRAINT

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ABSTRACT
Bacterial diseases mainly due to vibriosis in Penaeid shrimp culture implicating several species of Vibrios. *Vibrio harveyi* is considered as an important causative agent of the systemic vibriosis, which occurs in any bio-fields. The virulence of this bacterium is due to the production and expression of several virulent factors such as hemolysin, cysteine and metalloprotease, phospholipases, exotoxins, luciferase and siderophore. The present study intends the virulence determination among three strains of *V. harveyi* VSH3, VSH5 and VSH9 isolated from shrimp hatchery along the coastline of Tuticorin, Tamil Nadu during vibriosis outbreaks through their haemolysis and growth performance. Haemolytic activity examined against sheep blood resulted minor or no variations among the organisms in agar plate assay. However, in microtitre assay, Extra Cellular Products (ECP) of all isolates rendered increased activity with increase in dose and time of exposure. The bacterium VSH5 exhibited more haemolytic effect (at 500 µl concentration 72% haemolysis) than that of VSH9 and VSH3 reavled that the bacterium is highly virulent than the others. The bacterium VSH5 showed well growth in the optimum temperature (33°C), NaCl (2%) and pH (7.3). The maximum luminescence was expressed in 37°C, 2.5-3% NaCl and pH 7-9 in 18 to 48 hrs. Diverse colony morphology was observed in VSH5 on solid medium incubated for 3 to 5 days or longer. Growth curve experiment revealed that the bacterim VSH5 is a fast grower, completed its log phase within 7 hrs. Pathogenic strain like *V. harveyi* VSH5 causes high mortality and affects aquaculture production in hatchery as well as pond level. Hence, control measure against this kind of bacterium is urgently needed for maintain the sustainability of shrimp aquaculture in India and other Asian country.

KEYWORDS: *Vibrio harveyi* VSH5, Haemolysis, Growth Parameters, Colony Variation, Growth Curve.

INTRODUCTION

Shrimp aquaculture is a most important industry in India and other Asian countries. Shrimp hatcheries along the coastline involved in shrimp seed production often suffer enormous economic losses due to luminescent bacterial disease, commonly called as vibriosis. Vibriosis outbreaks are being increasingly recognized as a significant constraint to aquaculture production and trade in worldwide. The genus *Vibrio* belongs to the gamma-proteobacteria and is Gram-negative, usually motile rods (Thompson *et al*., 2004). *Vibrio* disease in aquaculture is described as vibriosis or bacterial disease, Penaeid bacterial septicaemia, Penaeid vibriosis, luminescent vibriosis or red-leg disease (Aguirre-Guzmán *et al*., 2004). Among the Vibrios, *Vibrio harveyi* (luminous *Vibrio*) is the main cause of shrimp death infecting larva in the hatchery as well as in the cultivation pond (Won & Park, 2008). *V. harveyi* is one of the important etiological agents of mass mortalities of *Penaeus monodon* larvae rearing systems. The virulence of *V. harveyi* causes 100% losses at a time in shrimp production (Chythanya *et al.*, 2002; Musa *et al*., 2008). Luminescent strains of *V. harveyi* have been reported to cause major losses in the shrimp larviculture in the Phillippine (Lavilla-Pitogo *et al*., 1990), Australia (Pizzutto & Hirst, 1995), South America (Alvarez *et al*., 1998; Robertson *et al*., 1998) and Mexico (Vandenberghe *et al*., 1999). Although almost all types of cultured crustaceans can be affected by these bacteria, the most serious problems have been reported in Penaeid shrimp culturing (Austin & Zhang, 2006). Adult shrimps suffering vibriosis may appear hypoxic, shows reddening of the body with red to brown gills, reduce feeding and may be observed swimming lethargically at the edges and surface of ponds (Anderson *et al*., 1988; Nash *et al*., 1992). Vibriosis infected post larvae (PL) exhibits reduced motility, reduced phototaxis and empty guts (Chen, 1992). Vibriosis is expressed by a way of number of syndromes which include oral and enteric vibriosis, appendage and cuticular vibriosis, localised vibriosis of wounds, shell disease, systemic vibriosis and septic hepatopancreatitis (Lightner, 1996). Vibriosis Infected animals shows signs of lethargy, tissue and appendage necrosis, slow growth, slow metamorphosis, body malformation, bioluminescence, muscle opacity and melanisation (Aguirre-Guzmán *et al*., 2004). Despite its role as a serious pathogen of cultured marine animals, the pathogenic mechanisms of *V. harveyi* yet have to be fully elucidated (Austin & Zhang, 2006), although several different virulent factors have been identified. Some of the hatcheries along the coastal regions of Tuticorin, South Tamil Nadu showed the symptoms of luminous vibriosis during the middle of 2012. The usage of medication and other treatments could not prevent the disease prevalence in the hatcheries. In our previous study we isolated several

vibrio pathogens during Vibriosis outbreak from the above said regions. Among the isolates, three (VSH3, VSH5 and VSH9) were phenotypically and genotypically identified as *V. harveyi*. Hence, the present study was performed to select the highly virulent strain among them based on their hemolytic and growth ability.

MATERIALS & METHODS

Bacterial strains

Three strains of *V. harveyi* VSH3, VSH5 and VSH9 were isolated and identified from hatchery water during vibriosis outbreak. The stock cultures maintained in Microbial Biotechnology Laboratory, Manonmaniam Sundaranar University, Alwarkurichi were used as target pathogens throughout the study.

Hemolysin test on agar plates

Hemolysin test was carried out according to the method described by Austin *et al*. (2005) with slight modification. Bacterial strains were grown overnight in marine broth at 25ºC in an incubator shaker. A drop of each of the isolate was spotted onto freshly prepared blood agar (marine agar containing 1% de-fibrinated sheep blood) plates. Finally, the plates were covered with parafilm and kept in an incubator at 30ºC for 48 hrs. The test was repeated three times.

Haemolytic activity of ECP

Extra cellular products (ECP) of the Vibrios were obtained by centrifuging overnight culture in a concentration of 1.5×10^8 cells/ml and tested for haemolytic activity. Haemolytic titrations were conducted in 96-well microtitre plates (Tarsons, Kolkata). Five ml of blood was collected from a healthy universal donor individual (O^{+ve}) and erythrocytes were collected after repeated washing in sterile normal saline (0.85% w/v NaCl, pH 7.2) and resuspended in normal saline to 0.5%. A volume of 0.5ml of the cell suspension was mixed with various concentrations of ECP (50, 100, 150, 200……450 and 500µl). Total volume of each well was made up to one ml with normal saline. The mixtures were incubated for 1 hr at 37ºC. Haemolytic activity was determined by the appearance of lytic erythrocytes visibly or microscopically. Haemolytic activity was also performed by spectroscopic method (Yang *et al*., 2005). Following incubation at 37ºC for 1 hr, the mixture was centrifuged at 1500 rpm for 10 min in a cooling centrifuge. The free hemoglobin in the supernatants was measured in UV-Visible spectrophotometer at 540 nm. Drabkin's solution (500 µl) and Saline (500 µl) were used as positive (maximal) and negative (minimal) haemolytic controls. Each experiment was performed in triplicates for each concentration.

The percentage haemolysis was calculated according to the following formula:

$$\% \text{ Haemolysis} = [A_t – A_n / A_c – A_n] \times 100$$

Where, A_t is the absorbance of test sample, A_n is absorbance of the negative control, A_c is the absorbance of the positive control.

Optimal condition for culturing highly virulent strain of *V. harveyi*

The bacterium was cultured in both solid and liquid medium of TSA and TSB. Colony morphology was studied on TCBS and marine agar. The bacterium was incubated at various temperatures (from room temperature to 37°C), in media of various percentages of NaCl (between 0.5-7%) and various alkalinity (pH 5 -12). Under these physical conditions growth, colony morphology and luminescence production were investigated.

Growth curve of virulent strain

About 200 ml of the marine broth was inoculated with the overnight culture of test organism and kept on Orbital shaker at room temperature. Observations for growth OD was taken using a spectrophotometer at 600nm of absorbance after every 15 min till the log phase was achieved, after which readings were taken every 30 minutes. Zero reading was calibrated with un-inoculated broth (control). Growth curve was plotted as OD 600 readings against time and different phases of growth were hence determined.

RESULTS

Haemolytic activity in plate and microtitre assay

In order to study the haemolytic activity (hemolysin production), certain volume of bacterial suspension of each isolate was spot inoculated on the plates supplemented with sheep blood. Finally opalescence or clearing zones on plates were noted relative to colony diameter. Haemolytic activity of each Vibrios were examined against sheep blood and the result indicated minor or no such variations occurring between the organisms (Table 1). Haemolytic activities of the ECP of isolated Vibrios were screened against normal human erythrocytes. All the three ECP indicated haemolytic effect. The results do not clearly report upon the visual observation of haemolysis in microtitre plate except negative control (button formation occurrence) but on microscopic observation it is clear that the VSH5 showed best haemolysis than the others. However the haemolytic percentage increased with increase in dose and time of exposure. Overall, VSH5 exhibited more haemolytic effect than that of VSH9 and VSH3. The high haemolytic activity of VSH5 (at 500 µl concentration 72% haemolysis) suggests that the bacterium is highly virulent in nature than the others (Fig. 1). Hence it was selected for further studies.

TABLE 1. Haemolytic activity towards sheep red blood of three isolates of *V. harveyi*

Strain	Zone of Clearance (mm)	Colony diameter (mm)	Ratio
V. harveyi VSH3	20.8±0.2	15.4±0.1	5.3±0.2
V. harveyi VSH5	20.9±0.5	15.1±0.1	5.8±0.5
V. harveyi VSH9	23.1±0.2	17.5±0.2	5.6±0.4

Results are expressed as mean ± standard deviation of three replicates

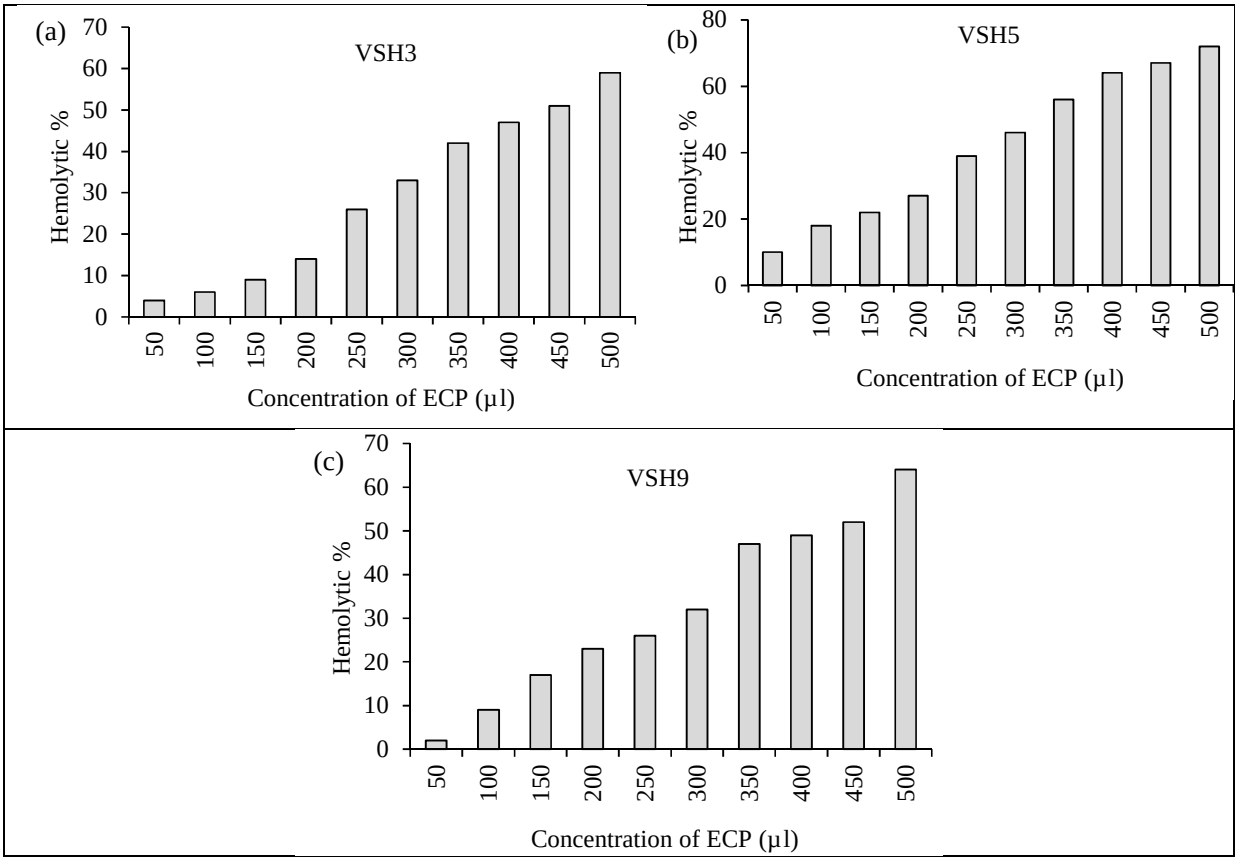

FIGURE 1. Percentage haemolytic activity of culture supernatant (ECP) from isolated *V. harveyi* (a) VSH3 (b) VSH5 and (c) VSH9

Optimal condition for culture of *V. harveyi* VSH5

V. harveyi VSH5 grew well from room temperature (25-30°C) to 35°C, but the best temperature for the growth was 33°C. It tolerated but did not grow well at 37°C or above. At higher temperatures like 37°C, it exhibited strong luminescence, but little or none at temperatures from room temperature to 33°C. However, luminescense weakened after incubation for 4-5 days, regardless of the incubation temperature. The luminescense could be reactivated in approximately 6 hrs after switching the cultures from a lower to a higher temperature (e.g., 30°C to 37°C). If the temperature was lowered once again, the luminescence faded. Salinity also influenced the growth of *V. harveyi* VSH5. It grew well in 2-3% NaCl but also persisted in percentages of NaCl from 1% upto 6.5%. It grew well between pH 7-9, but not at pH lower than 5 and slowly at any pH above 9. Also, at pH 7-9 it exhibited stronger luminescence than at lower pH (Table 2).

TABLE 2. Optimized conditions for the growth and culturing of *V. harveyi* VSH5

Parameters	Optimal condition for good growth	Tolerance persistence
Temperature	33°C	37°C but growth limited above the temperature
NaCl	2% NaCl	Persist between low (1% NaCl) and high (6.5% NaCl)
pH	pH 7.3 (pH 7-9)	Slow growth in low (pH 5) and high (above pH 9), beyond this level growth limited
Luminescence	37°C, 2.5-3% NaCl and pH 7-9 in 18 to 48 hrs	Weakened or faded luminescence in below 36°C, at low (below 2.5% NaCl) and above 3% NaCl and pH (below 7 & above 9) for up to 4 days

Variation in colony morphology and growth performance

Vibrio harveyi VSH5 sub-cultured on TCBS and marine agar showed variable colonial morphology. The appearance of the colonies was diverse in size, shape and color (Fig. 2). Though it appeared diverse in colony morphology, performed test reports each colony as biochemically identical. This kind of colony variation

appeared only on solid medium incubated for 3 to 5 days or longer. The variation was not evident upon daily subculture. Growth curve experiments revealed that VSH5 is a fast growing bacterial isolate that reached its log phase after 30 min of incubation at room temperature with continuous shaking of 150 rpm. The log phase continues up to 7.0 hrs after which the stationary phase seemed to start (Fig. 3).

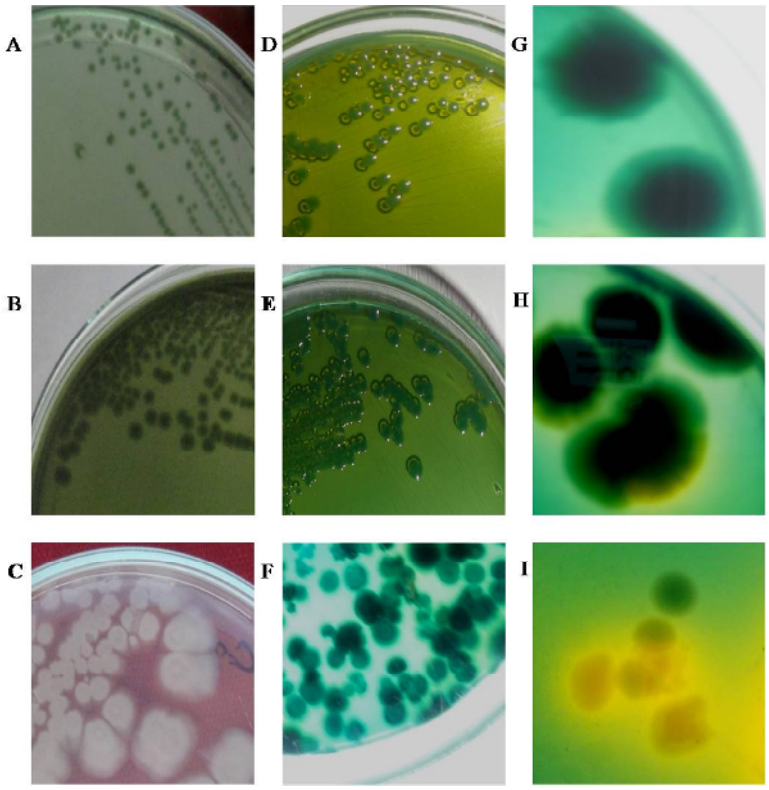

FIGURE 2. Various colony types of VSH5: (A) clouded green small rough colonies on TCBS (B) Smooth colonies with black pigments on TCBS (C) Small and big margin colonies on marine agar (D) Small dark centered yellow halo around colonies (E) Clear dark centered green colonies (F) Rough medium sized green unshaped colonies (G) large colonies with dark green round (H) Large elevated dark green to yellow colonies and (I) Colonies partially digested.

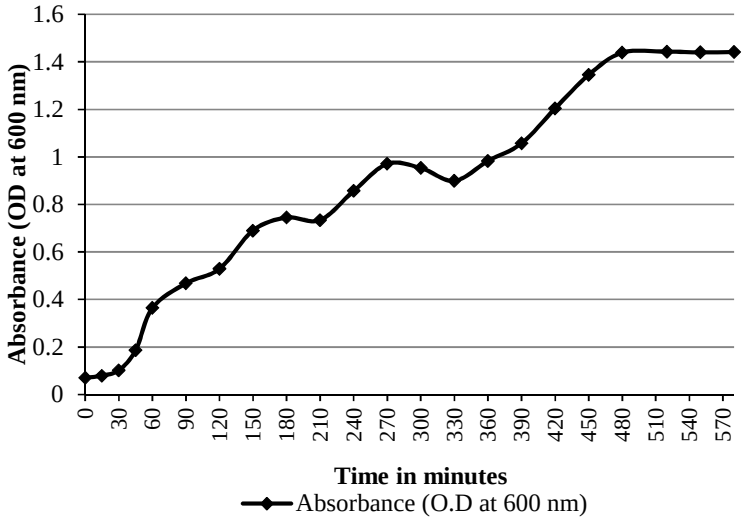

FIGURE 3. Growth curve of *V. harveyi* VSH5 in Marine broth

DISCUSSION

Nakayama *et al*. (2006) observed that the ECP of *V. harveyi* VP1 showed higher haemolytic activity compared with VT2 and other strains. A similar trend was also observed with the strain of *V. harveyi* VSH5 isolated in the present study. Several reports have evaluated the pathogenicity of environmental isolates of *V. harveyi* to larva and juvenile penaeid shrimps (Harris & Owen, 1999;

Pizzutto & Hirst, 1995; Ruangpan *et al*., 1999). One of the reasons for variation in the virulence levels of Vibrios reported in this study revealed that infectivity of *V. harveyi* is dependent on the virulence factors of the strains employed (Gomez-Gill *et al*., 1998). Some studies indicate that the virulence factors produced by *V. harveyi* can be contributed from toxins (either protease or hemolysin) (Liu & Lee, 1999; Zhang & Austin, 2000; Zhang *et al*., 2001). However, other studies represented that the pathogenicity of *V. harveyi* is derived from phage in which genes coding for toxin production are acquired by gene transduction (Morris & Robert, 1995). The toxin production in bacteria may be controlled by gene transduction but some bacteria have been found to express toxin by a process called quorum sensing (Bernd *et al*., 2001; Costi *et al*., 2002). It is reported that the bacterial luminescense is produced by different autoinducer in each genus or species. The major autoinducer of *V. harveyi* has been reported to be a long chain aliphatic aldehyde. Lux gene expression triggers the synthesis and accumulation of autoinducer during the growth of bacteria. The electron transport proceeds by the catalytic reaction of luciferase among the reduced flavin mononucleotide ($FMNH_2$), O_2 and a long chain aliphatic aldehyde produces flavin mononucleotide (FMN) and an aliphatic carboxylic acid which emits the light (Fisher *et al*., 1995). The results of the present study suggested that the temperature may also influence the expression of luminescence which is in accordance with the study of Pasharawipas *et al*., (1998). The temperature may either stimulate luciferase activity or the production or function of the autoinducer. The bacterium VSH5 does not grow at higher temperatures such as 37°C and above as the temperature may affect the production and activities of luciferase or autoinducer. The present study also reports that the luminescence expression was affected by pH. Optimum pH (7-9) resulted in strong luminescence which also correlates with the study of Pasharawipas *et al*. (1998). The combination of temperature and alkalinity might find some application in manipulation of *V. harveyi* for higher production of poly-3-hydroxybutyrate (PHB), a raw material in plastic industry due to its properties like thermoplasticity, water resistance and biodegradability. It was previously reported that the production of PHB is related to luminous expression controlled by the lux autoinducer (Sun *et al*., 1994).The variation in colony morphology of VSH5 is of somewhat interest. Similar variation was obtained by Pasharawipas *et al*.(1998) during sub-culturing of *V. harveyi* VH1039 on TSA and TCBS. The variability of VSH5 might involve the fact that it is a lysogenic host of temperate phages which are rarely found in culture environment. The variability of other bacteria has also been reported to be due to transposon like behaviour of bacteriophages (Reidl & Makalanos, 1995; Belas *et al*., 1984). This may be the best explanation for variable morphology of bacterial colonies since the biochemical tests did not change for each colony. The growth curve result of strain VSH5 is in corroboration with the findings of Aisha & Nuzhat (2011) who recorded the growth curve of *V. harveyi* N6. Mortality among the cultured shrimp in hatcheries is due to the presence of highly virulent strain like *V. harveyi* VSH5 with different colony morphology and growth performance. This proves that there is an urgent need for a new eco-friendly preventive measure against the *Vibrio* pathogens in shrimp aquaculture to overcome quality seeds (larvae) exports.

Study to determine the virulence levels of the bacterial pathogen in aquatic animals is a key to prevent vibriosis in marine aquaculture. The virulence of *V. harveyi* is reported dependent on host species (Vera *et al*., 1992), doses, time exposure and age of host species (Jun & Huaishu, 1998) and pathogenic factors of the bacterial strains (Gomez-Gill *et al*., 1998). This paper describes the virulence of three strains of *V. harveyi* isolated from shrimp hatchery water based on their haemolytic activity and growth performance. Highly virulent strain like VSH5 causes mortality on animals and affects aquaculture production in hatchery level. Hence, control measure against this kind of bacterial pathogen is urgently needed for sustainability of shrimp aquaculture in India and other Asian country.

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