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PROGNOSIS OF BIOFILM FORMATION OF *VIBRIO* BACTERIA ON SHRIMPS AND DIAGNOSIS OF VIBRIOSIS

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Abstract

Vibriosis, is a bacterial infection responsible for death of shrimps across the world, caused by different species and strains of the genus *Vibrio.* A large number of shrimp hatcheries involved in shrimp seed production often suffer setbacks due to luminescent bacterial disease and suffer enormous economic losses.

Keywords: Vibriosis, shrimp, Vibrio harveyi, P. monodon, luminescent bacterial disease.

INTRODUCTION

Vibriosis is ubiquitous and almost all marine shelled organisms, including shrimps, are susceptible. The disease has been reported prevalent in *P. monodon*, *P. japonicus* and *P. vannamei*, and is caused by a number of Vibrio species, *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. penaeicida.* There have been rare reports of vibriosis caused by *V. damsela*, *V. fluvialis too*. The exoskeleton of the shrimps tends to be an effective physical barrier to pathogens trying to penetrate their external surfaces. The *Vibrio* spp. are among the chitinoclastic bacteria and are capable to enter through wounds in the exoskeleton or pores. The gills are covered by a thin exoskeleton and appear susceptible to bacterial penetration. The mid-gut is not covered by an exoskeleton and thus seems to be a site for penetration of pathogens.

The initial step in the infection involves the adsorption of *Vibrio* to the Chitin or the cells. The Biofilm formation of this *Vibrio* is not studied so far. In this paper we have tried to study the mechanism of Biofilm formation i.e. adsorption of bacteria on the Shrimp and find out the possible mechanism to avoid the infection. Moreover this paper highlights the pathology, clinical signs, transmission and diagnostic methods to study Vibrosis.

Biofilms as already described, are well structured microbial communities with embedded within a mainly polysaccharide extracellular matrix. Biofilms can be defined as communities of microorganisms attached to a surface. The vast majority of biofilms cells are irreversibly attached to a substrate and its sessile cell components. These microorganisms in biofilms display phenotypes that are markedly different from that of planktonic or free floating cells[79-81]. Both gram-positive and gram-negative microorganisms have been isolated from the indwelling medical devices. Some of the commonly found species of gram-negative and gram-positive are *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermis, Streptococcus viridance and Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa* respectively.

Courtesy: KAU Agri-Infotech portal

It is not only difficult but also impossible to treat microorganism forming biofilms with antimicrobial agents in the body as detachment from the device may result in infection. Despite the broad knowledge about functional characteristics of adhesins promoting foreign-body colonization only little is known about the relative importance of the distinct molecules in different clinical settings.

Shrimps- Indian Market

India's seafood exports, including frozen shrimps, stood at $3.5 billion in the 2012-13 fiscal. About a fifth of the exports were to the U.S. The Vannamei variety of shrimps played a major role in boosting India's marine products export during the 2012-13 fiscal. The export of this shrimp variety, cultivated in the East Coast, fetched $730 million against $385 million last year. India exported 91,000 tons of Vannamei shrimps in last fiscal against 40,787 tons the previous year, the Marine Products Export Development Authority sources said adding that the total shrimp exports comprising tiger, scampi and Vannamei during the period was 2.2

lakh tons (1.8 lakh tons). Marine product exports data for 2012-13 are yet to be released by the MPEDA. Last year, the country had garnered export revenue of Rs 19,000 crore ($3.5 billion). This year, MPEDA is expecting a five per cent increase in quantity and 12 per cent in value terms. Official sources at the Seafood Exporters Association of India said that Vanammei exports registered a considerable increase in the last three years, thanks to production by aqua farms in Andhra Pradesh. Exports were 12,407 tons in 2010-11 and 40,787 tons in 2011-12. Farmers in the East Coast region earned Rs 300 a kg in the last fiscal. The productivity of this variety is about 10 tons a hectare in the region. There has been a considerable increase in acreage under Vannamei farming and the total area under cultivation was 22,715 hectares. SEAI officials expressed the hope that shrimp exports from the East Coast will do better in the current year also on account of a good yield in aquaculture farms in Andhra Pradesh. This coupled with the detection of Early Mortality Syndrome for shrimps in Thailand, Vietnam, Malaysia and China will benefit India considerably.

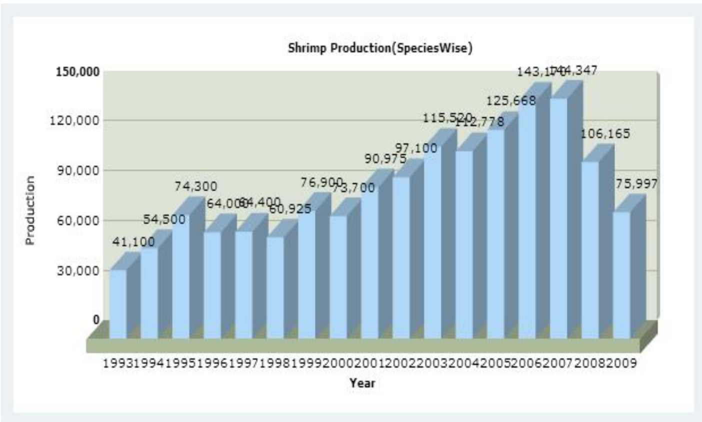

Fig. 2 Shrimp Production (Yearly) in India

Courtesy: **Central Institute of Brackishwater Aquaculture (CIBA)**

Present status of vibriosis

Vibriosis is a common problem world-wide, particularly in India. *V. harveyi* continues to cause chronic mortalities of up to 30% among *P. monodon* larvae, postlarvae and adult under stressful conditions. Highly pathogenic strains of Vibrio sp. are also emerging and continue to cause mortalities among cultured shrimp [28]. Problems caused by secondary vibriosis are common, but are considered minor compared to viral epidemics.

Pathogen Information

Vibriosis is caused by gram-negative bacteria in the family Vibrionaceae. Vibrio species are part of the natural marine flora and become opportunistic pathogens when the defence mechanisms are suppressed. Some strains of certain Vibrio species have been identified as primary pathogens. Pathogenic strains *of V. harveyi, V. vulnificus* and *V. parahaemolyticus* have caused massive epidemics in Thailand, the Philippines, India and Japan. *V. anguillarum*, *V. campbelli*, *V. nereis* and *V. splendidus* have also been associated with disease outbreaks in shrimps.

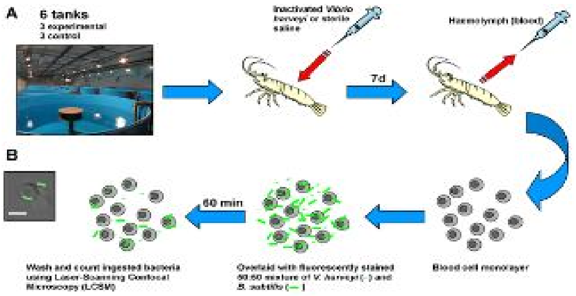

Fig. 3: Phagocytosis Experimental Design
Courtesy: Enhanced Cellular Immunity in Shrimp (*Litopenaeus vannamei*) after vaccination, Edward C. Pope, et al.

Vibrio harveyi, a gram-negative, luminous bacterium, is one of the important etiologic agents of release exotoxins and causes 80% mortality in *P. monodon* larval rearing systems. Among the *Vibrio harveyi* isolates, few are virulent, suggesting a great deal of molecular and genetic variation in this group of bacteria.

Clinical signs

High mortalities usually occur in young juvenile shrimps, and have been reported in market sized *P. monodon* [4]. Mortalities due to vibriosis occur in shrimps when they are affected by factors like, poor water quality, crowding, high water temperature, low DO and low water exchange [29; 30; 7]. *P. monodon* larvae showed mortalities within 48 hr of immersion with *V. harveyi* and *V. splendidus* [24]. Adult infected shrimps may appear hypoxic, with red to brown gills, reduce feeding and may be observed swimming lethargically at the edges and surface of ponds [4; 40]. Vibrio spp. also cause red-leg disease, characterized by red coloration of the pleopods, periopods and gills, in juvenile and adult shrimps. Also, mortality rate increase up to 95% during the warm temperatures of the water [9]. *V. cholerae* has been reported to cause eyeball necrosis disease [9].

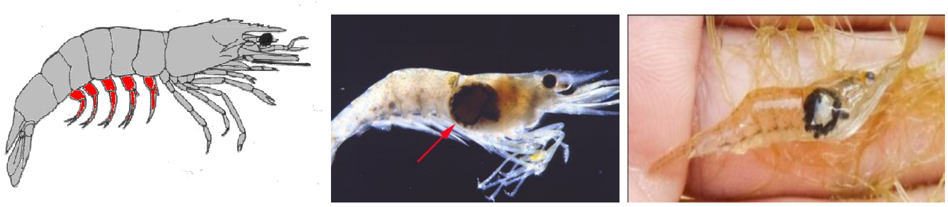

Fig. 4 Red-Leg Disease, Necrosis in gills & eyes of shrimps

Courtesy: www.inaturalist.org

The luminescence caused by *V. harveyi* and *V. splendidus* in infected postlarvae, juveniles and adults is readily visible at night [47; 31]. Infected postlarvae may also exhibit reduced motility, lowered phototaxis and empty guts [9].

Pathology

The symptoms of vibriosis include oral and enteric vibriosis, shell disease, appendage and cuticular vibriosis, localized vibriosis of wounds, systemic vibriosis and septic hepatopancreatitis.

Vibriosis infected shrimps may display localized lesions on the cuticle and gills, loss of limbs, cloudy muscles, localized infections in the gut, hepatopancreatic infections and septicemia [49; 32]. Infected post-larvae show misty hepatopancreatitis and gills appear brown [51; 4]. Septic hepatopancreatitis is characterized by multifocal necrosis and haemocytic inflammation.

Vibrio sp. cause high mortality in shrimps by eliminating the epithelium and the peritrophic membrane, the two layers that protect the shrimp from infections. Also, loss of the epithelium may affect the regulation of water and ion uptake into the body [38; 41].

Transmission

Vibrio species exist in the water used in shrimp culture facilities [24] and the biofilm, which is formed on different water contact structures of hatcheries and farms. Bacteria enter shrimps via wounds or cracks in the cuticle and are ingested with food [45; 24]. The primary source of *V. harveyi* in hatcheries appears to be the midgut contents of female brood-stock, which are shed during spawning [25].

Viability

Many studies have been conducted regarding the effect of freezing on vibrio which might contaminate harvested shellfish. *V. vulnificus* in harvested oysters (*Crassostrea virginica*) survived storage at -20 ^{o}C for 70 days [44]. *V. parahaemolyticus*, isolated from homogenates of the oyster was found to be in inactivated state within 16 days at -15 ^{o}C when the bacterial load was high [10; 37]. There is recent evidence to suggest that *V. harveyi* can survive in sediments present in the ponds even after chlorination or treatment with lime [23].

Histopathology

Systemic vibriosis typically results in the formation of septic nodules in the lymphoid organ, heart and connective tissues of the gills, hepatopancreas, antennal gland, nerve cord, tendon and muscle [4; 36; 21]. Infected hepatopancreatic cells may have very low number of vacuoles, indicating low lipid and glycogen concentrations [4]. Vibriosis in *P. monodon* is related with the formation of spheroids in the lymphoid organ [40].

Diagnosis

Diagnosis of vibrio infection is based on clinical signs and the identification of rod-shaped Vibrio bacteria in lesions or nodules. Excised organs and nodules may be streaked on TCBS or general marine agar plates. When investigating post-larvae, the whole animal may be crushed and then streaked. Luminescent colonies may be observed after 12 to 18 hr if incubated at 25 to 30^{o}C. Vibrio isolates may be identified by: Gram staining, motility test, an oxidase test, mode of glucose utilisation, growth in the presence of NaCl, nitrate reduction and luminescence. Antimicrobial sensitivity tests may be used to identify vibriosis and can be run using the Minimum Inhibitory Concentration (MIC) method [33] or the Kirby-Bauer disk method (1986).

		Growth in broth:		Tests				
		With no NaCl added	1% NaCl	Oxidase	Nitrate Nitrite	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase
Group & Species								
1	*V. cholerae*	+	+	+	+	-	+	+
	V. mimicus	+	+	+	+	-	+	+
2	*V. metschnikovii*	-	+	-	-	V	V	-
3	*V. cincinn-atiensis*	-	+	+	+	-	V	-
4	*V. hollisae*	-	+	+	+	-	-	-
5	*V. damsela*	-	+	+	+	+	V	-
	V. fluvialis	-	+	+	+	+	-	-
	V. furnissi	-	+	+	+	+	-	-
6	*V. alginolyticus*	-	+	+	+	-	+	V
	V. para-haemolyticus	-	+	+	+	-	+	+
	V. vulnificus	-	+	+	+	-	+	V
	V. carchariae	-	+	+	+	-	+	-

Table 1. Adapted from Koneman's Color Atlas and Textbook of Diagnostic Microbiology, 6th Ed.

Mechanism of biofilm formation

A biofilm consists of bacterial cells immobilized in a substratum which is frequently embedded in an organic polymer matrix of microbial origin. Biofilms appear in many different forms, including layer, clump ridges, and even more complex micro colonies that are arranged into stakes or mushroom like formation[55,56]. Understanding the mechanism of biofilm formation has been fundamental importance in design of new biomaterials able to prevent biofilm growth on their surface[54-57, 59, 60].

The basic formation of biofilm can be described as a three stage process:

a. Initial microbial adhesion

The process starts with the attachment of bacteria to the substratum. Bacterial growth and division then lead to the colonization of the surrounding area and formation of biofilm[89-90]. The force to which bacteria are subjected to any separation distance is the sum of the Van der Waals forces, electrostatic forces, acid-base interactions and Brownian motion forces. In particular Van der Waals forces are generally attractive and result from induced dipole interaction between molecules in the colloidal particle and molecules in the surface[56,57]. A common approach to interpret initial microbial adhesion to non-conducting surface such as polymers and glass is understood by Derjaguin, Landau, Verway and Ocebeek (DLVO) theory. The cells attach to the surface by various mechanisms as given in Table 2. This step is a reversible phenomenon.

b. Irreversible microbial attachment and biofilm maturation

The former organisms, primary colonizers shift from reversible to irreversible attachment by the involvement of specific and selective binding between bacterial adhesions and substratum receptor. Once colonization has begun, the biofilm grows through a combination of cell division and recruitment[65-67]. It is the phase in which biofilm matures and there may be a change in its size and shape, depending on the organism and type of antimicrobial and experimental system, biofilm bacteria can be up to a thousand times more resistant to antimicrobial stress than free-swimming bacteria of the same species.

The generation of complex architecture characterizing mature biofilm has been shown to be under genetic control. Quorum sensing (QS), which is a cell density dependent bacterial intracellular signaling mechanism, enables bacteria to coordinate the expression of certain genes[57, 58]. The role of QS system in related strains is studied by interrupting cell to cell communication by the use of quorum sensing inhibitors such as furanose compounds [59-61]. These studies reveal interesting strategies to inhibit biofilm formation. DNA microarray results (Whitley et al, 2001) showed that over 70 unexpressed immature biofilms were genes encoding protein involved in translation, metabolism membrane transport and gene regulation[56-59, 70].

c. Cell aggregate detachment from mature biofilms

Cell aggregate detachment has recently been considered as a biofilm development process detachment is supposed to have some benefits for biofilm development [57]. Dispersal of cells from the biofilm colony is also an essential stage of the biofilm life cycle. Dispersal of cells enables biofilms to spread and to colonize new surfaces [70, 71].

Several enzymes such as dispersin B and deoxyribonuclease are found to play a major role in biofilm dispersal process. Biofilm matrix degrading enzymes are very important as they can be used as anti-biofilm agents. Recent studies have shown that cis-2-decenoic acid (a fatty acid messenger), is capable of inducing dispersion and inhibiting growth of biofilm colonies. This compound is secreted by *Pseudomonas aeruginosa* and induces cyclo heteromorphic cells in several species of bacteria and the yeast *Candida albicans*. Nitric oxide has also been shown to trigger the dispersal of biofilms of several bacteria species at sub-toxic concentrations. Nitric oxide has the potential for the treatment of patients that suffer from chronic infections caused by biofilms [72, 73].

Treatment

Vibriosis is controlled by rigorous water management and sanitation to prevent the entry of vibrio in the culture water [5] and to reduce stress on the shrimps [32]. Good site selection, pond design and pond preparation are also important [40]. An increase in daily water exchanges and a reduction in pond biomass by partial harvesting are recommended to reduce mortalities caused by vibriosis. Draining, drying and administering lime to ponds following harvest is also recommended [4].

Luminescent vibriosis may be controlled in the hatchery by washing eggs with iodine and formaldehyde. *V. harveyi* in the water column can be inactivated by chlorine dioxide. Probiotics (BioRemid-Aqua) are administered directly into the water or via feeds. Immuno-stimulants have had success in reducing shrimp mortalities associated with vibriosis.

Nakayama. T. et al (2007) investigated the effect of copper concentration on the expression of both luminescence and toxin of *V. harvey*. They found, *V. harveyi* cultured with 40 ppm copper concentration showed decreased luminescence, while, copper concentration of less than 40 ppm had no effect on the growth of shrimps. Therefore, the combination of prebiotics, probiotics, immuno-stimulants and non-antibiotic substances has superior specificity against vibriosis and luminescent bacteria coupled with Best Aquaculture Practices (BAP), which makes it an effective management tool for the control of luminescence bacterial toxicity in aquaculture.

CONCLUSIONS

From this review, it should be evident that some *V. harveyi* and *V. penaeicida* strains isolated from moribund shrimp may be true pathogens and be the primary cause of disease. Results of immersion challenges with these isolates indicate that the *Vibrio* densities used for the calculation of the lethal dose 50 may be naturally encountered in the shrimp rearing water. This hypothesis is reinforced by the finding of Sung et al., 1999, who observed in seawater from *P. monodon* cultured pond a decrease in the diversity of the *Vibrio* community associated with a dominance of few potentially virulent *Vibrio* species prior to outbreaks of vibriosis. Similar results were obtained by Lavilla-Pitogo et al., 1998, monitoring the bacterial population in the rearing water of several ponds cultured *P. monodon*. A dominance of luminescent *Vibrio* in rearing water of infected ponds was observed prior to outbreaks due to luminescent *Vibrio*. The virulence studies using the IM way of infection are more difficult to analyze in terms of epidemiological significance. There is a need for reproducible and standardized experimental models in order to evaluate the virulence of *Vibrio* isolates associated with mortalities, to test prophylactic and curative treatments and study the host-factors influencing the expression of bacterial virulence. As pointed out in this review, standardization of each step of pathogenicity tests is crucial because many environmental parameters, bacteria and shrimp factors may influence the results of a pathogenicity experiment.

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