

DRUG DEVELOPMENT FOR SHRIMP ACUTE HEPATOPANCREATIC NECROSIS DISEASE (AHPND)

Submitted in partial fulfilment of the requirements for the award of Bachelor of
Technology degree in Biotechnology.

By

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INSTITUTE OF SCIENCE AND TECHNOLOGY
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of R. SUKRIYA (38230036) who carried out the project entitled DRUG DEVELOPMENT FOR SHRIMP ACUTE HEPATOPANCREATIC NECROSIS DISEASE (AHPND) under my supervision from November to march.

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DECLARATION:

I **R. SUKRIYA (38230036)** hereby declare that the Project Report entitled “**Drug Development for Shrimp Acute Hepatopancreatic Necrosis Disease (AHPND)**” done by me under the guidance of **Dr. M. Masilamani Selvam (Internal)** at **Sathyabama Institute of Science and Technology** is submitted in partial fulfilment of the requirements for the award of **Bachelor of Technology degree in Biotechnology**.

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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) or formerly known as early mortality syndrome (EMS) is an emerging disease that has caused significant economic losses to the aquaculture industry. The primary causative agent of AHPND is *Vibrio parahaemolyticus*, a Gram-negative rod-shaped bacterium that has gained plasmids encoding the fatal binary toxins Pir A/Pir B that cause rapid death of the infected shrimp. In this review, the current research studies and information about AHPND in shrimps have been presented. Molecular diagnostic tools and potential treatments regarding AHPND were also included. This review also includes relevant findings which may serve as guidelines that can help for further investigation and studies on AHPND or other shrimp diseases.

AHPND is formerly known as early mortality syndrome (EMS) for it can cause sudden, mass mortalities of up to 100% in shrimps that can be observed within 30–35 days of stocking. This disease shows greater susceptibility in *Penaeus monodon* (Asian tiger shrimp) and *L. vannamei* (white leg shrimp). The involvement of these shrimp species leaves devastating economics losses that reached about billions of dollars annually since the first outbreak in China at 2009. *Vibrio parahaemolyticus*, the causative agent of AHPND, is a Gram-negative rod-shaped bacterium that is ubiquitous in nature. AHPND-causing *V. parahaemolyticus* is detrimental to shrimps but not to humans. This bacterium produces a thermolabile hemolysin (TLH) diagnostic marker negative of both human toxigenic genes: thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH). The pathogenic bacteria contain unique extrachromosomal plasmids that are not found in non-pathogenic strains. The transposon-like insert is unrelated to AHPND and shows no difference on virulence between the two groups even if it is found on virulence plasmids.

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ABBREVIATION:

ABBREVIATION	FULLFORM
AHPND	Acute Hepatopancreatic Necrosis Disease
TCBS	Thiosulfate-Citrate-Bile Salts-Sucrose agar
EMS	Early Mortality Syndrome
PEG	Poly Ethylene Glycol

1. INTRODUCTION:

Aquaculture industry has a big role in global economy. This provides job opportunities and revenue to business sectors. Its demand increases throughout the years and is very much dependent on human consumption. Among the different areas of aquaculture, crustacean industry has developed rapidly in the past years due to the increase market demands of crustaceans all over the world. Prawn and shrimp industries have been considered major revenue generators, and many aquaculturists focus on cultivating shrimps and prawns as primary protein sources for consumption.

Shrimp is considered one of the most important commodities that are being traded in terms of market value. Although there is an increased production of global farmed shrimp, leading producing countries, particularly Asia, have experienced a decline in production due to shrimp diseases.

One of the most crucial problems in aquaculture is the occurrence of different diseases risking the health and production of aquatic animals. Marine animals literally swim in potential pathogens making them susceptible to viruses, bacteria, fungi, and even protozoans. General epidemic outbursts induced by viruses, such as Taura syndrome virus (TSV), infectious hypodermal and hematopoietic virus (IHHNV), white spot syndrome virus (WSSV), hepatopancreatic parvovirus (HPV), yellow head virus (YHV), and monodon baculovirus (MBV), brought about 15 billion US dollars of losses to shrimp farmers for the past 15 years with 80% of such loss attributed to Asia alone. The outbreak of diseases can significantly reduce the economic growth and cultivation of different aquatic species.

Last 2017, a recent discovery of iridescent virus in China which caused high mortality and serious diseases in *Lit Penaeus vannamei* has been verified and termed as shrimp haemocyte iridescent virus. In addition to these lists, an infectious disease called acute hepatopancreatic necrosis disease (AHPND) has emerged with clinical signs of severe atrophy of the shrimp hepatopancreas and accompanied by unique histopathology at the acute stage of the infection. AHPND is formerly known as early mortality syndrome (EMS) for it can cause sudden, mass mortalities of up to 100% in shrimps that can be observed within 30–35 days of stocking. Shortly after the discovery of *V. parahaemolyticus*, metagenomics sequencing was

done to provide insights on the disease mechanism in shrimps and develop diagnostic tools. It was also found that an AHPND-causing non-*V. parahaemolyticus* species *V. owenii* and a strain close to it possessed pVA1-like plasmids which implies that these toxin genes can be transferred to various species of *Vibrio*. It can be hypothesized that these genes are flanked in the plasmid by mobile genetic elements which could be a transposase-coding sequence that may be potentially involved in horizontal gene transfer.

The pVA1 plasmid contains two plasmid mobilization genes and a group of transfer genes for conjugation indicating that the plasmid may be self-transmissible. Bacterial clades with high degree of similarity at both phenotypic and genotypic levels have demonstrated gene transfer through recombination events, transposition, conjugation, and plasmid uptake during infection period. These processes explain the huge possibility of conversion from non-pathogenic to pathogenic strain and positively enhance the spread of AHPND. Furthermore, *V. parahaemolyticus* AHPND strains from Mexico (13-511/A1 and 13-306D/4) were found to be carrying *tab* gene coding tetracycline resistance gene and *V. campbellii* (Vc3S01) from China was found to be carrying multiple antibiotic resistance genes.

These results lead to failure of disease control if traditional methods of disease management and eradication will be employed for AHPND. The global production of cultured crustaceans for 2018 is predicted to be ~8.63 million tonnes, and shrimp represents a significant portion of cultured crustaceans. Demands for shrimp are raising, also in developing countries as consumer preferences have evolved with rising incomes. Shrimp is mainly produced for consumption but during processing economically valuable products are being generated such as chitosan, which is used in cosmetics, food and beverages, agrochemicals and pharmaceuticals. However, the potential and sustainability of shrimp aquaculture production currently suffer from high mortality rates, losses due to especially bacterial and viral infections, and hence suboptimal yields.

Vibrio parahaemolyticus acute hepatopancreatic necrosis and white spot syndrome virus (WSSV) disease outbreaks are examples of economically devastating disease outbreaks in shrimp. There is a need for prophylaxis of

infectious diseases and for finding novel strategies for conditioning economically important shrimp broods so that they can produce more robust progenies and develop tolerance against biotic (pathogens) and abiotic stressors. Training the innate immune system of shrimp could be a possible avenue for improving animal's health, and it might serve as potential unique strategy for conditioning the brood stock to have more robust progenies.

The current review provides an overview on innate immune responses in shrimp after exposure to bacteria, viruses and abiotic stressors and on the possibilities to train the innate immune system of shrimp in order to improve health, not only of the animal itself but also of its progeny. Fishes and other aquatic foods play a pivotal role to achieve nutrition and food security goals. Aquaculture is also being considered to be one of the most promising food-producing sectors with the largest potential to accomplish growing demand of food.

If the chronological trends related to income and population growth, urbanization, and diets remain conserved, then demand for seafood will increase extensively over the years (2030–2050). Furthermore, seafood is claimed to evidently provide more environmentally sustainable animal-source foods. Crustaceans serve as an important aquatic food commodity, and among the crustaceans, the shrimps constitute the most significant group that contribute to the global fishery sector. Shrimp and prawns have been recognized as most heavily traded fish products. Asia and Latin America have lion's share in bulk production of shrimps while USA, the European Union, and Japan are the major consumers of shrimps. The Global Aquaculture Alliance's GOAL (Global Outlook for Aquaculture Leadership) 2019 survey depicted global shrimp aquaculture production with reference to different regions in the part of the world over a decade from 2010 to 2021 summarizes estimated global production of shrimps and reported a surge in shrimp production in 2018 which would further escalate through 2021. As per [businesswire.com](https://www.businesswire.com), the global shrimp market faced deterioration due to COVID-19 lockdown but still it was enumerated to be US\$ 18.30 billion in 2020 and is expected to be US\$ 23.4 billion by 2026.

2. LITERATURE SURVEY:

S.NO	Year	Title	Author
1	2000	Homologue of the crustacean Daphnia is diversified by alternative splicing like in insects.	Brits D, McTaggart S, Morris K, Anderson J, Thomas K, et al.
2	2000	Penaeus monodon has a highly diverse cytoplasmic tail and is the first membrane-bound shrimp.	Chou P-H, Chang H-S, Chen I-T, Lee C-W, Hung H-Y, et al.
3	2000	Evidence for the presence of subpopulations of Arenicola marina coelomocytes identified by their selective response towards Gram+ve and Gram-ve bacteria.	Fitzgerald SW, Ratcliffe NA
4	2001	Zhang SM, Ademar CM, Kepler TB, Looker ES. Diversification of Ig superfamily genes in an invertebrate.	Zhang SM, Ademar CM, Kepler TB, Looker ES.
5	2001	Strain-specific priming of resistance in the red flour beetle, Trifolium castoreum.	Roth O, Sad BM, Schmid-Hempel P, Kurtz J.
6	2002	Schmid-Hempel P. Trans-generational immune priming in a social insect.	Sadd BM, Hempel Schmid-Hempel R
7	2003	'Trans-generational immune priming': specific enhancement of the antimicrobial immune.	Moreot Y.

8	2004	Phagocytosis mediates specificity in the immune defence of an invertebrate, the woodlouse <i>Porcellio scaber</i> (Crustacea: Isopoda). <i>Dev Comp Immunol.</i>	Roth O, Kurtz J.
9	2005	Crustacean immunity. <i>Adv Exp Medical.</i>	Cerenius L, Jiravanichpaisal P, Liu HP, Sidehill I
10	2006	prawn <i>Macrobrachium rosenbergii</i> (de Man) and its resistance against <i>Aeromonas hydrophila</i> infection and nitrite stress.	Chand RK, Sahoo PK, Kumari J et al
11	2008	Treatment of infected open wounds on two dogs using a film dressing of polysaccharide extracted from the hulls of durian.	Chansiripornchai P, Pongsamart S
12	2008	Changes in haemocyte counts in <i>L. stylirostris</i> subjected to sublethal infection and to vaccination.	C. Goarant, E. Boglio
14	2009	Summer Syndrome in <i>stylirostris</i> in New Caledonia Pathology and epidemiology of the etiological agent, <i>V. nigrispulchritudo</i> .	C. Goarant, D. Ansquer, J. Herlin
15	2009	Assessment of microbial dynamics and antioxidant enzyme.	L.T. Guzmán-Villanueva, C. Escobedo-Fregoso

16	2010	The involvement of crustaceans toll-like receptors in pathogen recognition Fish Shellfish Immunol.	Y.J. Habib, Z. Zhang
17	2010	Molecular detection of Enterocytozoon <i>hepatogenic</i> and <i>V. parahaemolyticus</i> -associated acute hepatopancreatic necrosis disease in Southeast Asian <i>P. vannamei</i> shrimp	J.E. Han, S.C. Lee, S.C.
18	2011	Occurrence of bacteria species responsible for vibriosis in shrimp pond culture systems in Sri Lanka and assessment of the suitable control measures.	P.P.M. Heenatigala, M.U.L. Fernando
19	2011	Isolation of the bacterium, <i>V. harveyi</i> from cultured shrimp, <i>P. monodon</i> and production of vaccines against the bacterium.	M. Hettiarachchi, S. Pathirage, G. Hettiarachchi
20	2012	Efficacy of vaccination against vibriosis in cultured kuruma prawns <i>P. japonicus</i> .	T. Itami, Y. Takahashi, Y. Nakamura
21	2013	Variation in <i>V. parahaemolyticus</i> isolates from a single Thai shrimp farm experiencing.	J. Joshi, J. Srisala, V.H. Truong

22	2014	Mass mortality of <i>P. monodon</i> larvae due to antibiotic-resistant <i>V. harveyi</i> infection.	I. Karunasagar, R. Pai, G.R. Malahti, I. Karunasagar
23	2014	<i>Vibrio</i> Bacterin and carboxymethyl β -1,3-glucans protect <i>monodon</i> from <i>V. harveyi</i> infection	S.S. Klannukarn, K. Wongprasert, K. Khanobdee, P. Meeratana, P. Taweepreda.
24	2016	Ultrastructural and cytochemical characteristics of phagocytes in kuruma prawn Fish Pathol.	M. Kondo, T. Itami, Y. Takahashi, R. Fujii, S.
25	2016	Truncated VP28 as oral vaccine candidate against WSSV infection in shrimp: an uptake and processing study in the midgut of <i>P. Monodon</i> .	A. Kulkarni, J.H.W.M. Rombout, I.S.B. Singh, N.S. Sudheer, J.M. Vlak, C.M.A. Caipang, M.F. Brinchmann, V. Kiron
26	2017	Feed based vaccine in aquaculture.	M. Kumar, G. Gupta, Vikas, S. Sharma
27	2018	Innate defence: evidence for memory in invertebrate immunity.	J. Kurtz, K. Franz
28	2019	Bacterial diseases in tiger shrimp culture in the Philippines.	C.R. Lavilla-Pitogo, L.D. de la Pena
29	2019	Early events in crustacean innate immunity.	S.Y. Lee, K. Soderhall
30	2019	Tiger shrimp penaeid in possesses semi-granulocyte adhesion.	C. Li, H. Yan, Y. Song

31	2019	Characterize a typically Dscam with alternative splicing in mud crab <i>Scylla paramamosain</i> .	W. Li, X. Tang, Y. Chen, W. Sun, Y. Liu, Y. Gong, X. Wen.
32		Virus diseases of farmed shrimp in the Western Hemisphere.	D.V. Lightner
33	2020	A. Mandal, S.K. Das Comparative efficacy of neem and non-neem supplemented media in controlling the harmful luminescent bacteria in natural pond culture.	A. Mandal, S.K. Das
34	2020	Fine structure and classification of shrimp haemocytes.	G.G. Martin, B. Graves
35	2020	Immune memory in invertebrates.	B. Milinkovic, J. Kurtz
36	2021	Worms and flies as genetically tractable animal models to study host-pathogen interactions.	E. Mylonakis, A. Aballay
37	2021	Vaccination trials with <i>P. japonicus</i> to induce resistance to white spot syndrome.	A. Namikoshi, J.L. Wu, T. Yamashita, T. Nishizawa, T. Nishioka, M. Arimoto
39	2021	Dscam in immunity: a question of diversity in insects and crustaceans.	T.H. Ng, J. Kurtz
40	2021	Review of Dscam-mediated immunity in shrimp and other arthropods.	T.H. Ng, Y.-A. Chiang, Y.-C. Yeh, H.-C. Wang

3. AIM:

Production of a drug for acute hepatopancreatic necrosis disease in shrimp. Acute hepatopancreatic necrosis disease (AHPND) or formerly known as early mortality syndrome (EMS) is an emerging disease that has caused significant economic losses to the aquaculture industry. The primary causative agent of AHPND is *Vibrio parahaemolyticus*, a Gram-negative rod-shaped bacterium. The presence of pathogenic bacteria in the worldwide marine environment raises concerns of human on food safety due to the latter potentially causing disease outbreaks depending on the environmental conditions. A good example is *Vibrio parahaemolyticus*, a member of *Vibrio* species from the *Vibrionaceae* family. *V. parahaemolyticus* is a Gram-negative halophilic bacterium that is widely disseminated in estuarine, marine and coastal surroundings. *V. parahaemolyticus* is usually found in a free-swimming state; with its motility conferred by a single polar flagellum affixed to inert and animate surfaces including zooplankton, fish, shellfish or any suspended matter underwater. The classification of *V. parahaemolyticus* depends on the antigenic properties of the somatic (O) and capsular (K) antigen produced in various environmental condition. *Vibrio parahaemolyticus* was first discovered by Tsunesaburo Fujino in 1950 as a causative agent of food borne disease following a large outbreak in Japan which recorded 272 illnesses with 20 deaths after consumption of shirasu. Virulent *V. parahaemolyticus* strains are transmitted by consumption of raw or undercooked seafood causing acute gastroenteritis.

3.1 SCOPE:

- To study about the early mortality syndrome in shrimp
- Collection of shrimps, feed, water for sampling
- Isolation of vibrio from shrimp
- Providing the drug against the early mortality against shrimp.

4. MATERIAL:

4.1 ISOLATION AND IDENTIFICATION OF VIBRIO PARAHAEMOLYTICUS:

Several selective agar media have been devised, but thiosulfate citrate bile salts sucrose (TCBS) agar is recommended for the isolation of *V. parahaemolyticus*. In clinical microbiology laboratories, MacConkey agar containing 0.5% additional NaCl is also convenient for routine culture of diarrheal stools.

Enrichment culture is used for the detection of the vibrio from food and marine samples. Polymyxin salt broth, containing 2% NaCl and 50 µg ml⁻¹ of polymyxin B (pH 7.6) may be used for the selective growth of *V. parahaemolyticus*. It should be noted that some factor(s) in shellfish may inhibit the growth of vibrios. It is therefore recommended that shellfish are cut into small pieces, but not homogenized. After shaking the enrichment broth vigorously, the pieces of shellfish are removed with forceps. However, enrichment culture of seafoods incriminated in outbreaks of infection may be unrewarding, because most yield Kanagawa-negative isolates, in contrast to those from patients.

The colonial appearance of *V. parahaemolyticus* on TCBS agar is so typical that provisional identification of the isolates from stool specimens may be made directly from the plates. However, isolates from marine sources must be further examined in order to differentiate them from related organisms. The addition of 1% NaCl to medium for biochemical tests is essential to obtain valid reactions.

The Kanagawa reaction is a reliable test for the recognition of virulent strains. For determination, isolates should if possible be tested which contains 0.5% yeast extract, 1% peptone, 0.5% mannitol, 0.05% K₂HPO₄, 7% NaCl, 0.0001% crystal violet, 1.5% agar, and washed human red blood cells. However, as adequate sources of human blood, or of blood from other suitable animals, are not always readily available, other suitable media may be considered. Thus, an enzyme-linked immunosorbent assay has been described for the detection of TDH-producing vibrios.

Serotyping of isolates of *V. parahaemolyticus* may be performed by slide agglutination tests using O and K antisera. In outbreaks of *V. parahaemolyticus* infection, however, the same serovar as that identified in patients is seldom detected in incriminated seafoods. Serotyping of isolates from seafoods and marine sources is thus not usually significant unless they are Kanagawa-positive. 4.2 requirements for Oxidase test.

Moist filter paper with the substrate (1% tetramethyl-p-phenylenediamine dihydrochloride), or commercially prepared paper disk, wooden wire, or platinum wire. Kovacs oxidase reagent (1% tetra-methyl-p-phenylenediamine dihydrochloride, in water). Store refrigerated in a dark bottle for no longer than 1 week. Soak a small piece of filter paper in 1% Kovac's oxidase reagent and let dry.

Use a loop and pick a well-isolated colony from a fresh (18- to 24- hour culture) bacterial plate and rub onto treated filter paper. Observe for colour changes. Oxidase positive: colour changes to dark purple within 5 to 10 seconds. Delayed oxidase-positive: colour changes to purple within 60 to 90 seconds. Oxidase negative: colour does not change or it takes longer than 2 minutes.

4.2 MODEL ORGANISM:

E. coli is frequently used as a model organism in microbiology studies. Cultivated strains. *coli* K12 are well-adapted to the laboratory environment, and, unlike wild-type strains, have lost their ability to thrive in the intestine. Many laboratory strains lose their ability to form biofilms. These features protect wild-type strains from antibodies and other chemical attacks, but require a large expenditure of energy and material resources. *E. coli* is often used as a representative microorganism in the research of novel water treatment and sterilisation methods, including photocatalysis. By standard plate count methods, following sequential dilutions, and growth on agar gel plates, the concentration of viable organisms or CFUs (Colony Forming Units), in a known volume of treated water can be evaluated, allowing the comparative assessment of materials performance.

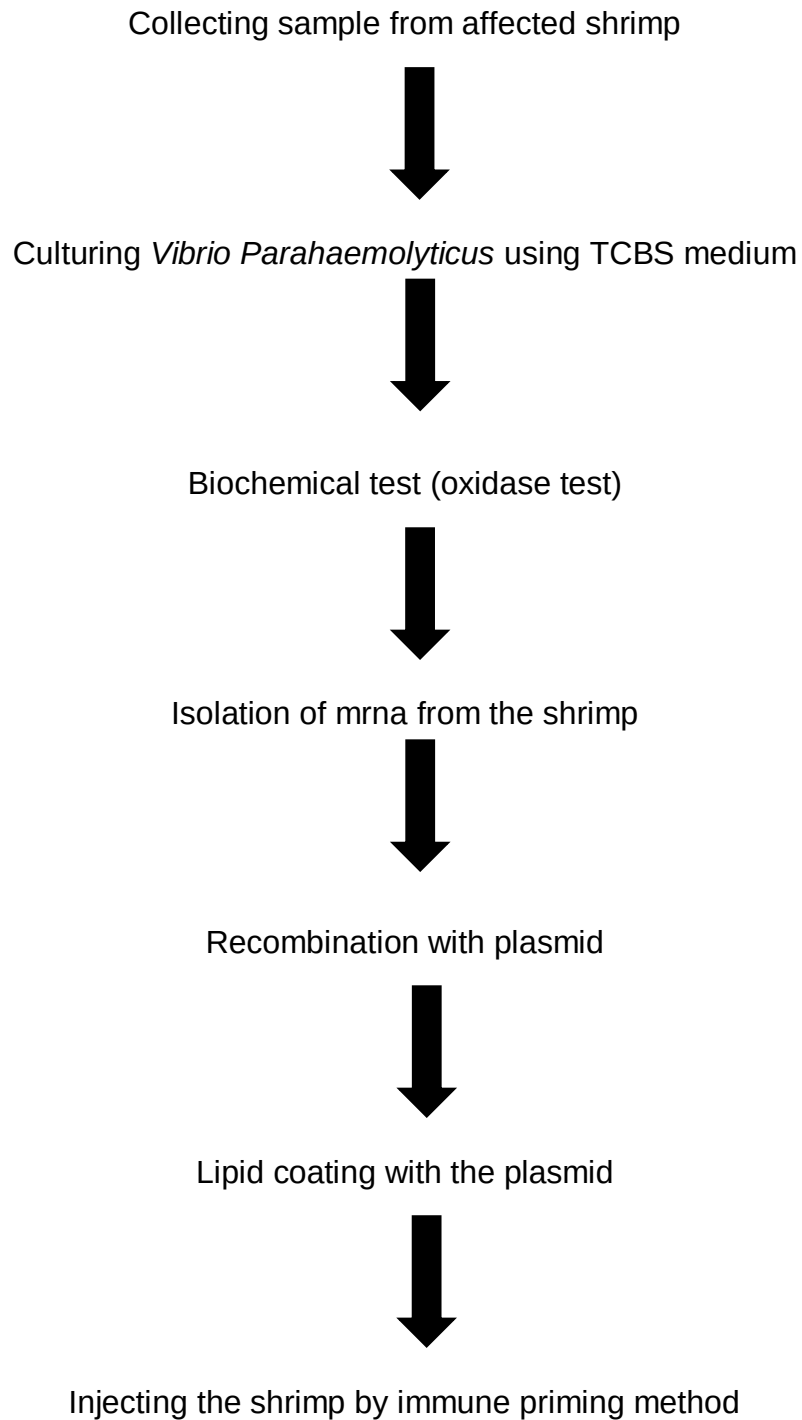
In 1946, Joshua Lederberg and Edward Tatum first described the phenomenon known as bacterial conjugation using *E. coli* as a model bacterium,

and it remains the primary model to study conjugation. coli was an integral part of the first experiments to understand phage genetics, and early researchers, such as Seymour Benzer, used E. coli and phage T4 to understand the topography of gene structure. Prior to Benzer's research, it was not known whether the gene was a linear structure, or if it had a branching pattern. coli was one of the first organisms to have its genome sequenced; the complete genome of E. coli K12 was published by science in 1997.

4.3 IMMUNE PRIMING:

Immune priming is a two-step vaccination technique wherein pathogens are introduced into the host's system followed by a secondary vaccination or the infection of the same pathogen. In crustaceans, immune priming in *L. vannamei* resulted in a high phagocytic activity against AHPND after introduction of recombinant viral protein from within spores of *Bacillus subtilis*. Immunity of *P. monodon* against vibriosis using prior vaccination with formalin-killed *Vibrio* showed longer day post-delivery survival. Improved immunity against bacterial pathogens was also observed on both post-larvae and larger juveniles of *L. vannamei*. Larger juveniles exposed to formalin-inactivated have boosted natural antibacterial activity by selective increase in phagocytosis and percentage of haemocytes. In shrimps, higher resistance or tolerance to pathogen from which the antigen was originally derived has been exhibited, suggesting that protection by immune priming is specific only to the type and strain of pathogen used during primary vaccination. Immune specificity responds more readily to native or natural pathogens that commonly infect the host. Its lack of response to other pathogens presents a lower number of immune receptors as compared with jawed vertebrates with millions of antibodies. Immune priming is then considered to be neither universal nor specific but rather a selective immunity.

5. METHOD:



5.1 SAMPLE COLLECTION:

Suspension made of shrimp samples and Phosphate Buffer Saline (PBS) were incubated for enrichment of *Vibrio* sp at 37°C for 18-24 h. Isolation of *Vibrio* sp was performed by streaking a loop of suspension on selective media viz. Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar. Following incubation, plates were examined for colony morphology. Single well-defined colony was further sub-cultured onto TCBS agar until pure colony was obtained. Gelatine agar (GA), Blood agar and MacConkey agar supplemented with 2% NaCl were used as the non-selective medium for purification of *Vibrio* sp. For further identification, the predicted *Vibrio* sp isolates were subjected to morphological test by Gram's staining and biochemical tests including oxidase test, carbohydrate fermentation tests, catalase test, MRVP test and indole test according to the procedures.

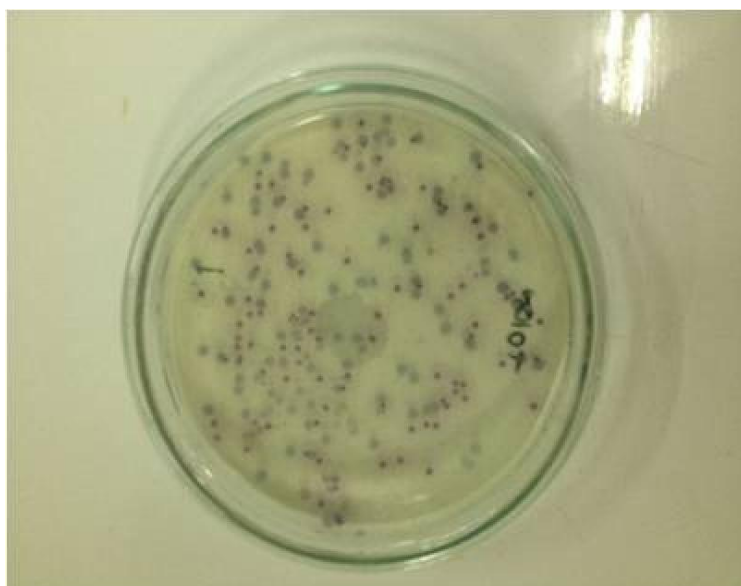


Fig: 5.1.1: Vibrio parahaemolyticus culture using TCBS agar.

5.2 COMPOSITION OF TCBS AGAR:

Table: 5.2.1 Composition of TCBS agar.

Sucrose	20.0 gm
Di peptone	10.0 gm
Sodium Citrate	10.0 gm
Sodium Thiosulfate	10.0 gm
Sodium Chloride	10.0 gm
Yeast Extract	5.0 gm
Ox bile (Ox gall)	5.0 gm
Sodium Cholate	3.0 gm
Ferric Citrate	1.0 gm
Bromothymol Blue	0.04 gm
Thymol Blue	0.04 gm
Agar	15.0 gm

5.3 BIOCHEMICAL OXIDASE TEST USING TCBS AGAR:

Conduct the oxidase test with fresh growth from an HIA slant or any non-carbohydrate containing medium. Do not use growth from thiosulfate citrate bile salts sucrose (TCBS) agar. Place 2 to 3 drops of oxidase reagent (1% tetramethyl-p-phenylenediamine) on a piece of filter paper in a petri dish. Smear the culture across the wet paper with a platinum (not nichrome) loop, a sterile wooden applicator stick, or toothpick. In a positive reaction, the bacterial growth becomes dark purple within 10 seconds. Color development after 10 seconds should be disregarded. Positive and negative controls should be tested at the same time. Organisms of the genera *Vibrio*, *Aeromonas*, *Plasmons*, *Pseudomonas*, and *Alcaligenes* are all oxidase positive; all *Enterobacteriaceae* are oxidase negative.



Fig: 5.3.1: Biochemical oxidase test for *vibrio parahaemolyticus*.

Moist filter paper with the substrate (1% tetramethyl-p-phenylenediamine dihydrochloride), or commercially prepared paper disk, wooden wire, or platinum wire. Kovacs oxidase reagent (1% tetra-methyl-p-phenylenediamine dihydrochloride, in water). Store refrigerated in a dark bottle for no longer than 1 week. Soak a small piece of filter paper in 1% Kovac's oxidase reagent and let dry.

Use a loop and pick a well-isolated colony from a fresh (18- to 24- hour culture) bacterial plate and rub onto treated filter paper. Observe for colour changes. Oxidase positive: colour changes to dark purple within 5 to 10 seconds. Delayed oxidase-positive: colour changes to purple within 60 to 90 seconds. Oxidase negative: colour does not change or it takes longer than 2 minutes.

5.4 ISOLATION OF mRNA FROM THE VIBRIO PARAHAEMOLYTICUS:

Total RNAs were extracted from infected shrimp hepatopancreas using TriReagent (Qiagen), and the mRNAs were purified from the total RNAs using the PolyATtract mRNA isolation systems (Promega) following the manufacturer's instructions. Equal amounts of the high-quality mRNA samples from each group were then pooled for cDNA synthesis and sequencing. Total RNA was isolated from the examined tissue (about 100 mg) of the shrimp using TRIzol reagent following the manufacturer's protocol, resuspended in DEPC-treated water and stored at -80 °C. The concentration of RNA was determined using a Nanodrop 2000 spectrometer, and RNA integrity was assessed by 1% agarose gel electrophoresis. The cDNA was synthesized from 1 µg of mRNA using a Prime Script Reverse Transcriptase kit following the manufacturer's protocol, as previously described. The cDNA was used as the template for PCR reactions in gene cloning. The phenol-chloroform-isoamyl alcohol method was used to isolate total genomic DNA, which was then used as template to amplify introns. A total of 43 sRNAs described in the Bacterial Small RNA Database (BSRD) for the pandemic *V. parahaemolyticus* were identified in 20 Chilean strains of *V. parahaemolyticus*. Nine of them, namely, svpa279.1 (Alpha_RBS), svpa788.1 (mini-ykkC), svpa996.1 (ffs), svpa1079.1 (Cyclic di-GMP-II riboswitch), svpa1191.1 (Lysine riboswitch), svpa1370.1 (Purine riboswitch), svpa2576.1 (S15), svpa3118.1 (P26), and svpa3233.1 (thiamin pyrophosphate (TPP) riboswitch), were highly conserved between pandemic and

nonpandemic strains, with 100% identity compared to the sRNAs described in reference strain RIMD2210633, of pandemic origin. Interestingly, svpa117.1 (Spot42) and svpa113.1 (RyhB), the two most studied sRNAs in *V. parahaemolyticus*, also showed high-percentage identity between pandemic and nonpandemic strains compared to the reference sequences.

Since we observed that some sRNAs were 100% conserved between nonpandemic strains and reference strain RIMD2210633, we investigated if these sRNAs were acquired by horizontal gene transfer (HGT) from pandemic to nonpandemic strains or vice versa. The criteria were first to determine if sRNAs and their genomic context were fully conserved between nonpandemic strains and the reference strain, and then to search for inverted repeat sequences, integrases, or transposases in the genomic context that could indicate HGT events. Analysis of the immediate genomic context (initially including 500 bp upstream plus 500 bp downstream) of 100% conserved between RIMD2210633 and nonpandemic strains (PMA12.14, PMA14.14, and PMA2.15), showed that there were single-nucleotide variants (SNV) in the flanking sequences, and only the sRNA sequence was 100% conserved between pandemic and nonpandemic strains. The percentage of identity calculated for the genomic context of nonpandemic strains compared to the RIMD2210633 strain was about 98–99%, as was expected for bacteria belonging to the same species. PMA37.5 was used as a Chilean pandemic strain control. As was expected, sRNAs and genomic context were fully conserved compared to the pandemic reference strain.

5.5 RECOMBINATION OF PLASMID BY CELL CULTURE TRANSFECTION:

Cell culture, transfection and fluorescence assays were carried out as described previously. Insect High Five cells (Invitrogen, USA) were cultured in a 96-well plate with Express Five serum-free medium (SFM) (Invitrogen, USA) containing L-glutamine (Invitrogen, USA) at 27°C. The cells in each well were co-transfected with 0.2 µg of the constructed plasmids and 100 NM of miR-1000 or miR-1000-scrambled with Cellfectin II Reagent (Invitrogen, USA) according to the manufacturer's instructions. All the miRNAs were synthesized by Shanghai Gene Pharma (Shanghai, China). At 48 h after the co-transfection, the fluorescence

intensity of cells was determined using a Flex Station II microplate reader (Molecular Devices, USA) at 490/510 nm excitation/emission (Ex/Em). Based on the fluorescence values of the cells co-transfected with miR-1000 and EGFP-wsv191-3'UTR, EGFP-wsv407-3'UTR or EGFP-wsv024-3'UTR, the relative fluorescence intensity was calculated. This assay was biologically repeated for three times.



Fig: 5.5.1: 96 well plate for cell transfection

The goal of stable, long-term transfection is to isolate and propagate individual clones containing transfected DNA that has integrated into the cellular genome. Distinguishing non-transfected cells from those that have taken up exogenous DNA involves selective screening. This screening can be accomplished by drug selection when an appropriate drug-resistance marker is included in the transfected DNA. Alternatively, morphological transformation can be used as a selectable trait in certain cases. For example, bovine papilloma virus vectors produce a morphological change in transfected mouse CI127 cells. Before using a particular drug for selection purposes, you will need to determine the amount of drug necessary to kill non-transfected cells. This amount may vary greatly among cell types and should be optimized for each new cell line being transfected. When drug selection is used, cells are maintained in nonselective medium for 1–2 days after transfection, then replated in selective medium containing the drug. The use of selective medium is continued for 2–3 weeks, with frequent changes of medium to eliminate dead cells and debris, until distinct colonies can be visualized. Individual colonies can be isolated by cloning cylinders, selected and transferred to multiwell plates for further propagation.

5.6 LIPID COATING WITH THE PLASMID:

PEG coating can prevent aggregation of LUVs induced by covalent coupling of protein to the surface of the LUVs and can inhibit fusion between LUVs. It was therefore of interest to determine whether the stabilizing properties of a PEG coating could prevent aggregation during dialysis. However, the use of the standard PEG-phosphatidylethanolamine (PEG-PE) was contraindicated because the PEG-PE molecule bears a net negative charge and could displace the cationic lipid from the plasmid, as has been noted for other negatively charged lipids. As a result, PEG₂₀₀₀ was linked to ceramide as the hydrophobic anchor to produce a neutral molecule. Two ceramide anchors were synthesized which differed in the length of the ceramide acyl chain (CerC₁₄ and CerC₂₀). When 10 mol% PEG-CerC₂₀ was incorporated in the detergent mixture with POPC, DODAC and plasmid DNA, precipitation was not observed during detergent dialysis. Further, a proportion of the plasmid was encapsulated. Lipid Encapsulation Enables the Effective Systemic Delivery of Polyplex Plasmid DNA. Using a new controlled mixing process, highly transfection-competent polyplexes were formed and subsequently encapsulated within a lipid bilayer. A detergent dialysis procedure is described which allows encapsulation of plasmid DNA within a lipid envelope, where the resulting particle is stabilized in aqueous media by the presence of a poly (ethylene glycol) (PEG) coating. These 'stabilized plasmid-lipid particles' (SPLP) exhibit an average size of 70 nm in diameter, contain one plasmid per particle and fully protect the encapsulated plasmid from digestion by serum nucleases and E. coli DNase I. Encapsulation is a sensitive function of cationic lipid content, with maximum entrapment observed at dioleoyl dimethylammonium chloride (DODAC) contents of 5 to 10 mol%. Two ceramide anchors were synthesized which differed in the length of the ceramide acyl chain (CerC₁₄ and CerC₂₀). When 10 mol% PEG-CerC₂₀ was incorporated in the detergent mixture with POPC, DODAC and plasmid DNA, precipitation was not observed during detergent dialysis.

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where the resulting particle is stabilized in aqueous media by the presence of a poly (ethylene glycol) (PEG) coating. The formulation process results in plasmid-trapping efficiencies of up to 70% and permits inclusion of 'mucigenic' lipids such as dioleoyl phosphatidylethanolamine (DOPE). The in vitro transfection capabilities of SPLP are demonstrated to be strongly dependent on the length of the acyl chain contained in the ceramide group used to anchor the PEG polymer to the surface of the SPLP. Shorter acyl chain lengths result in a PEG coating which can dissociate from the SPLP surface, transforming the SPLP from a stable particle to a transfection-competent entity.

5.7 INJECTING THE SHRIMP BY IMMUNE PRIMING METHOD:

Invertebrates, including shrimps, are believed to lack true lymphocytes and advanced humoral immune responses. They rely primarily on their innate or non-specific immunity as their primary and only protection against pathogens. Unlike vertebrates that have immune memory that easily recognizes specific pathogens by antigens and produces effector and memory cells through clonal expansion, the survival of non-adaptive invertebrates against microbial pathogens with generation times of just a few minutes was in question under the theory of modern evolution. Recently, studies about improved immunity of invertebrates after application of vaccine support possible existence of adaptive immunity.

Immune priming is a two-step vaccination technique wherein pathogens are introduced into the host's system followed by a secondary vaccination or the infection of the same pathogen. In crustaceans, immune priming in *L. vannamei* resulted in a high phagocytic activity against WSSV after introduction of recombinant viral protein from WSSV within spores of *Bacillus subtilis*. Immunity of *P. monodon* against vibriosis using prior vaccination with formalin-killed *Vibrio* showed longer day post-delivery survival. Improved immunity against bacterial pathogens was also observed on both post-larvae and larger juveniles of *L. vannamei*. Larger juveniles exposed to formalin-inactivated *V. harveyi* have boosted natural antibacterial activity by selective increase in phagocytosis and percentage of hemocytes. In shrimps, higher resistance or tolerance to pathogen from which the antigen was originally derived has been exhibited, suggesting that protection by

immune priming is specific only to the type and strain of pathogen used during primary vaccination. Immune specificity responds more readily to native or natural pathogens that commonly infect the host. Its lack of response to other pathogens presents a lower number of immune receptors as compared with jawed vertebrates with millions of antibodies. Immune priming is then considered to be neither universal nor specific but rather a selective immunity.

Further research studies are focusing on developing a vaccine against *V. parahaemolyticus* – causing AHPND. Since shrimps lack an adaptive immune system, a passive immunity could possibly protect shrimps from vibriosis by oral administration with specific egg yolk powders (IgY), and in vitro results showed that it can inhibit *V. parahaemolyticus* and *V. harveyi*. Related studies have done this type of passive immunization which demonstrated that the specific IgY effectively inhibited the growth of *V. parahaemolyticus* and provided passive immunity to shrimps. Recently, *V. parahaemolyticus* was attenuated by a gene knockout of LpxD gene responsible for the virulence of the bacteria and provided significant amount of protection in shrimps against challenge.

This method can be used to attenuate a virulent bacterium that may be employed as a live attenuated vaccine against *V. parahaemolyticus*. Since toxins A and B of *V. parahaemolyticus* can cause AHPND, monoclonal antibodies against toxins A and B were generated, and these monoclonal antibodies can be utilized to detect the toxins using dot blotting. Although the generated monoclonal antibodies were proven effective in detecting toxins A and B and as a therapeutic agent, they are not yet proven effective as a treatment/therapeutic agent against AHPND.

As of now, there are no commercial shrimp vaccines available against AHPND. But many studies tried to utilize the *V. parahaemolyticus* antigens to develop a vaccine against this pathogenic bacterium. Some studies used anti-Pir A-like toxin IgY in feeds and exhibited passive immunization against AHPND. This method of adding anti-Pir A-IgY in feeds could be a potent prophylactic means against AHPND infection. Further research studies are focusing on developing a vaccine against *V. parahaemolyticus*—causing AHPND. Since shrimps lack an adaptive immune system, a passive immunity could possibly protect shrimps from vibriosis by oral administration with specific egg yolk powders (IgY), and in vitro

results showed that it can inhibit *V. parahaemolyticus* and *V. harveyi*. Related studies have done this type of passive immunization which demonstrated that the specific IgY effectively inhibited the growth of *V. parahaemolyticus* and provided passive immunity to shrimps. Recently, *V. parahaemolyticus* was attenuated by a gene knockout of LpxD gene responsible for the virulence of the bacteria and provided significant amount of protection in shrimps against challenge. This method can be used to attenuate a virulent bacterium that may be employed as a live attenuated vaccine against *V. parahaemolyticus*. Since toxins A and B of *V. parahaemolyticus* can cause AHPND, monoclonal antibodies against toxins A and B were generated, and these monoclonal antibodies can be utilized to detect the toxins using dot blotting. Although the generated monoclonal antibodies were proven effective in detecting toxins A and B and as a therapeutic agent, they are not yet proven effective as a treatment/therapeutic agent against AHPND. The weakness or shortcoming that needs to be addressed is the vaccine development against *V. parahaemolyticus*. Vaccine progress against AHPND is a major issue since further studies to understand the underlying mechanisms and pathogenesis of the disease are needed to better understand the disease. Receptors, toxins, or genes responsible for the virulence of the disease combined with current knowledge and techniques currently available could make vaccine studies accelerate and further the AHPND studies.

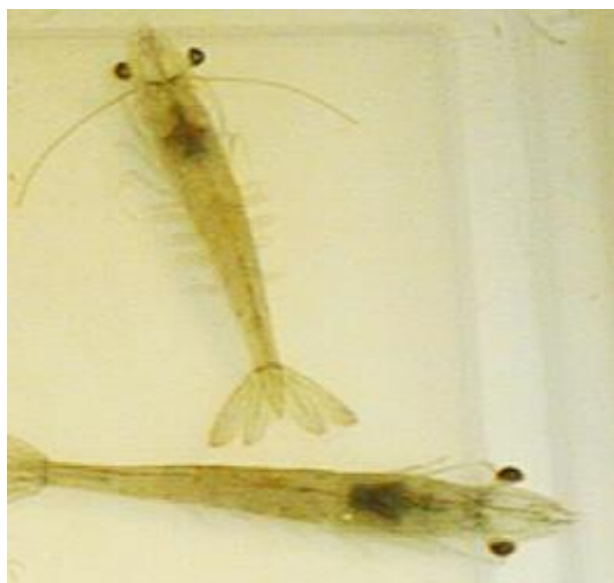


Fig: 5.7.1: Shrimps are alive after immune priming.

6. RESULT AND DISCUSSION:

Since the outbreak of AHPND in China back in 2009, control measures began and has been implemented to control and eradicate the disease. Many molecular diagnostic tools have been developed and implemented to reduce the effects of AHPND outbreak. Some of them have the potential to be used on-site to help the farmers diagnose AHPND, but the bigger issue remains on how do we prevent and treat AHPND. Several studies tackling on the development of therapeutic agents have the potential to control the disease and possibly prevent disease outbreaks. Since shrimps lack an adaptive immune response critical to combat infectious diseases like AHPND, further studies must be done to investigate the shrimp's immune responses and the underlying mechanisms. These would be essential in developing vaccines and treatments against AHPND and other shrimp diseases. Concerning AHPND, the Pir A- and Pir B-like receptors need to be investigated further, as interaction mechanisms with the host organisms along with the pathogenesis could be proven useful for AHPND prevention studies. Understanding the underlying mechanism of the disease and pathogenicity is crucial in preventing and eradicating AHPND. All experimentations regarding AHPND must follow laboratory safety and biosecurity protocols in order to ensure the safety of its people and prevent the spread of infectious substances to the environment related to AHPND. Considering AHPND as a recent emerging disease, biosurveillance, treatment, diagnostics, and management of this disease are great dilemmas faced by many.

6.1 SAMPLE COLLECTION:

Suspension made of shrimp samples and Phosphate Buffer Saline (PBS) were incubated for enrichment of *Vibrio* sp at 37°C for 18-24 h. Isolation of *Vibrio* sp was performed by streaking a loop of suspension on selective media viz. Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar. Following incubation, plates were examined for colony morphology. Single well-defined colony was further sub-cultured onto TCBS agar until pure colony was obtained. Gelatine agar (GA), Blood agar and MacConkey agar supplemented with 2% NaCl were used as the non-selective medium for purification of *Vibrio* sp. For further identification, the predicted

Vibrio sp isolates were subjected to morphological test by Gram's staining and biochemical tests including oxidase test, carbohydrate fermentation tests, catalase test, MRVP test and indole test according to the procedures.

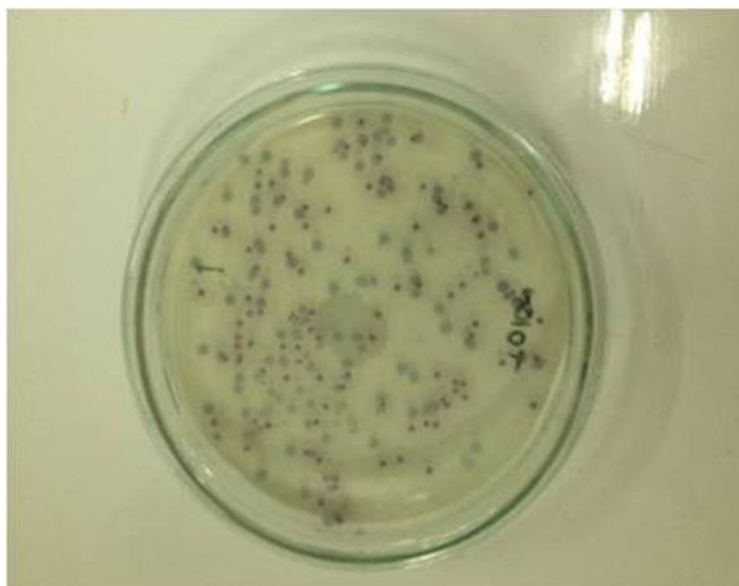


Fig: 6.1.1: *Vibrio parahaemolyticus* culture using TCBS agar.

6.2 COMPOSITION OF TCBS AGAR:

Table: 6.2.1 Composition of TCBS agar

Sucrose	20.0 gm
Di peptone	10.0 gm
Sodium Citrate	10.0 gm
Sodium Thiosulfate	10.0 gm
Sodium Chloride	10.0 gm
Yeast Extract	5.0 gm
Ox bile (Ox gall)	5.0 gm
Sodium Cholate	3.0 gm
Ferric Citrate	1.0 gm
Bromothymol Blue	0.04 gm
Thymol Blue	0.04 gm
Agar	15.0 gm

6.3 BIOCHEMICAL OXIDASE TEST:

Conduct the oxidase test with fresh growth from an HIA slant or any non-carbohydrate containing medium. Do not use growth from thiosulfate citrate bile salts sucrose (TCBS) agar. Place 2 to 3 drops of oxidase reagent (1% tetramethyl-p-phenylenediamine) on a piece of filter paper in a petri dish. Smear the culture across the wet paper with a platinum (not nichrome) loop, a sterile wooden applicator stick, or toothpick. In a positive reaction, the bacterial growth becomes dark purple within 10 seconds. Colour development after 10 seconds should be disregarded. Positive and negative controls should be tested at the same time. Organisms of the genera *Vibrio*, *Aeromonas*, *Plasmons*, *Pseudomonas*, and *Alcaligenes* are all oxidase positive; all *Enterobacteriaceae* are oxidase negative.

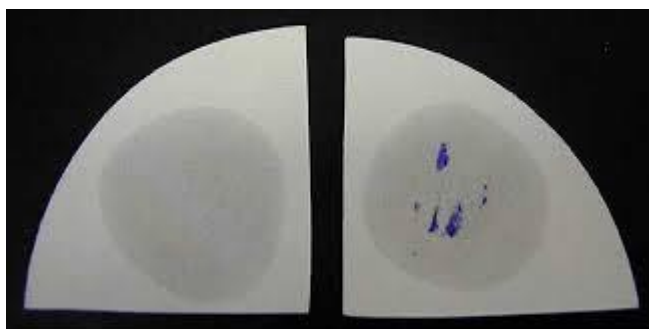


Fig: 6.3.1: Biochemical oxidase test for *vibrio parahaemolyticus*

6.4 ISOLATION OF mRNA FROM THE VIBRIO PARAHAEMOLYTICUS

Total RNAs were extracted from infected shrimp hepatopancreas using TriReagent (Qiagen), and the mRNAs were purified from the total RNAs using the PolyATtract mRNA isolation systems (Promega) following the manufacturer's instructions. Equal amounts of the high-quality mRNA samples from each group were then pooled for cDNA synthesis and sequencing. Total RNA was isolated from the examined tissue (about 100 mg) of the shrimp using TRIzol reagent following the manufacturer's protocol, resuspended in DEPC-treated water and stored at -80°C . The concentration of RNA was determined using a Nanodrop 2000 spectrometer, and RNA integrity was assessed by 1% agarose gel electrophoresis. The cDNA was synthesized from 1 μg of mRNA using a Prime Script Reverse Transcriptase kit following the manufacturer's protocol, as previously described. The

cDNA was used as the template for PCR reactions in gene cloning. The phenol-chloroform-isoamyl alcohol method was used to isolate total genomic DNA, which was then used as template to amplify introns. A total of 43 sRNAs described in the Bacterial Small RNA Database (BSRD) for the pandemic *V. parahaemolyticus* were identified in 20 Chilean strains of *V. parahaemolyticus*. Nine of them, namely, svpa279.1 (Alpha_RBS), svpa788.1 (mini-ykkC), svpa996.1 (ffs), svpa1079.1 (Cyclic di-GMP-II riboswitch), svpa1191.1 (Lysine riboswitch), svpa1370.1 (Purine riboswitch), svpa2576.1 (S15), svpa3118.1 (P26), and svpa3233.1 (thiamine pyrophosphate (TPP) riboswitch), were highly conserved between pandemic and nonpandemic strains, with 100% identity compared to the sRNAs described in reference strain RIMD2210633, of pandemic origin. The concentration of RNA was determined using a Nanodrop 2000 spectrometer, and RNA integrity was assessed by 1% agarose gel electrophoresis. The cDNA was synthesized from 1 µg of mRNA using a Prime Script Reverse Transcriptase kit following the manufacturer's protocol, as previously described. The cDNA was used as the template for PCR reactions in gene cloning. Interestingly, svpa117.1 (Spot42) and svpa113.1 (RyhB), the two most studied sRNAs in *V. parahaemolyticus*, also showed high-percentage identity between pandemic and nonpandemic strains compared to the reference sequences.

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Cell culture, transfection and fluorescence assays were carried out as described previously. Insect High Five cells (Invitrogen, USA) were cultured in a 96-well plate with Express Five serum-free medium (SFM) (Invitrogen, USA) containing L-glutamine (Invitrogen, USA) at 27°C. The cells in each well were co-transfected with 0.2 µg of the constructed plasmids and 100 NM of miR-1000 or miR-1000-scrambled with Collecting II Reagent (Invitrogen, USA) according to the manufacturer's instructions. All the miRNAs were synthesized by Shanghai Gene Pharma (Shanghai, China). At 48 h after the co-transfection, the fluorescence intensity of cells was determined using a Flex Station II microplate reader (Molecular Devices, USA) at 490/510 nm excitation/emission (Ex/Em). Based on the fluorescence values of the cells co-transfected with miR-1000 and EGFP-wsv191-

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encapsulated plasmid from digestion by serum nucleases and *E. coli* DNase I. Encapsulation is a sensitive function of cationic lipid content, with maximum entrapment observed at dioleoyl dimethylammonium chloride (DODAC) contents of 5 to 10 mol%. The formulation process results in plasmid-trapping efficiencies of up to 70% and permits inclusion of 'mucigenic' lipids such as dioleoyl phosphatidylethanolamine (DOPE). The in vitro transfection capabilities of SPLP are demonstrated to be strongly dependent on the length of the acyl chain contained in the ceramide group used to anchor the PEG polymer to the surface of the SPLP. Shorter acyl chain lengths result in a PEG coating which can dissociate from the SPLP surface, transforming the SPLP from a stable particle to a transfection-competent entity.

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Immune priming is a two-step vaccination technique wherein pathogens are introduced into the host's system followed by a secondary vaccination or the infection of the same pathogen. In crustaceans, immune priming in *L. vannamei* resulted in a high phagocytic activity against WSSV after introduction of recombinant viral protein from WSSV within spores of *Bacillus subtilis*. Immunity of *P. monodon* against vibriosis using prior vaccination with formalin-killed *Vibrio* showed longer day post-delivery survival. Improved immunity against bacterial pathogens was also observed on both post-larvae and larger juveniles of *L. vannamei*. Larger juveniles exposed to formalin-inactivated *V. Harvey* have boosted natural antibacterial activity by selective increase in phagocytosis and percentage of haemocytes. In shrimps,

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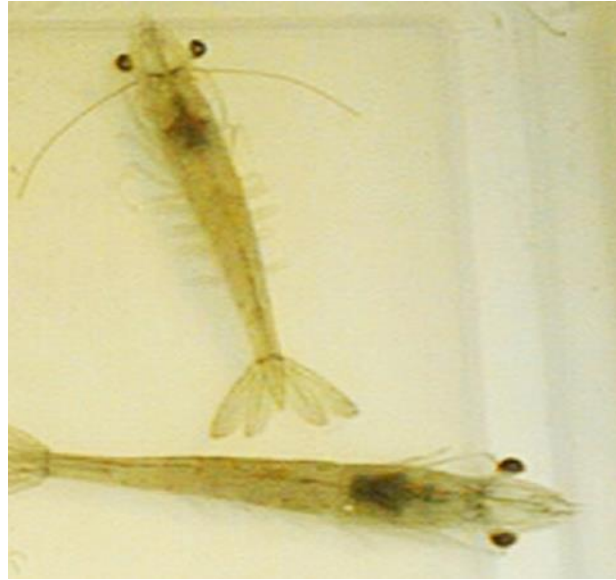


Fig: 6.7.1: Shrimps are alive after immune priming.

7. SUMMARY

Vibrio parahaemolyticus, the causative agent of AHPND, is a Gram-negative rod-shaped bacterium that is ubiquitous in nature. AHPND-causing *V. parahaemolyticus* is detrimental to shrimps but not to humans. Both *P. monodon* and *L. vannamei* with EMS present the same pathology. Samples of shrimp preserved for histology have shown the effects of EMS appear to be limited to the hepatopancreas (H.P.). Progressive dysfunction of the H.P. results from lesions that reflect degeneration and dysfunction of the tubule epithelial cells that progress from proximal to distal. This bacterium produces a thermolabile hemolysin (TLH) diagnostic marker negative of both human toxigenic genes: thermostable direct hemolysin (TDH) and TDH-related hemolysin. The pathogenic bacteria contain unique extrachromosomal plasmids that are not found in non-pathogenic strains.

There are two identified types of AHPND-causing *V. parahaemolyticus* bacteria based on geographical variations. Isolated *V. parahaemolyticus* in Mexico and Central USA showed a 4243-bp Tn3-like transposon insert at ORF4 which is not present on the Asian type (isolated from China, Vietnam, and Thailand). The first changes observed in the hepatopancreases of affected shrimp is a marked reduction of fat storage cell vesicles and loss of oil/fat droplets, as well as a decrease in the activity of secretory cells. As the disease progresses, fat, basophilic and secretory cells degenerate and begin to round up, detach from the H.P. tubule basement membrane and slough into the H.P. tubule lumen.

Concomitant with the degeneration of these cells in the more proximal regions of the H.P. tubules, the number of mitotically active E cells declines, and the marked inflammatory response is dominated by haemocyte infiltration and encapsulation of the affected H.P. tubules.

As the tubule epithelial cells degenerate, their nuclei become variably hypertrophic, and the nuclei of most nuclei become enlarged. In the terminal stages of the disease, a severe secondary infection likely caused by opportunistic *Vibrio* bacteria occurs in the sloughed masses of epithelial cells in the H.P. tubule lumens. Affected shrimp die from H.P. dysfunction and the terminal vibrio infection.

The transposon-like insert is unrelated to AHPND and shows no difference on virulence between the two groups even if it is found on virulence plasmids. The unique extrachromosomal plasmid found in pathogenic *Vibrio* species encodes the genes for AHPND's main virulence factors which are Pir A and Pir B toxins. Pathogenic effects of AHPND include atrophication and production of lesions despite detection of low population of bacteria. A study by [1] detected the presence of Pir A and Pir B toxins in protein lysates from the stomach, hepatopancreas, and haemolymph of diseased shrimps. But the bigger issue remains on how do we prevent and treat AHPND. Several studies tackling on the development of therapeutic agents have the potential to control the disease and possibly prevent disease outbreaks. Since shrimps lack an adaptive immune response critical to combat infectious diseases like AHPND, further studies must be done to investigate the shrimp's immune responses and the underlying mechanisms. These would be essential in developing vaccines and treatments against AHPND and other shrimp diseases. Concerning AHPND, the Pir A- and Pir B-like receptors need to be investigated further, as interaction mechanisms with the host organisms along with the pathogenesis could be proven useful for AHPND prevention studies. Understanding the underlying mechanism of the disease and pathogenicity is crucial in preventing and eradicating AHPND.

7.1 CONCLUSION:

Since the outbreak of AHPND in China back in 2009, control measures began and has been implemented to control and eradicate the disease. Many molecular diagnostic tools have been developed and implemented to reduce the effects of AHPND outbreak. Some of them have the potential to be used on-site to help the farmers diagnose AHPND, but the bigger issue remains on how do we prevent and treat AHPND. Several studies tackling on the development of therapeutic agents have the potential to control the disease and possibly prevent disease outbreaks. Since shrimps lack an adaptive immune response critical to combat infectious diseases like AHPND, further studies must be done to investigate the shrimp's immune responses and the underlying mechanisms.

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Aquaculture industry has a big role in global economy. This provides job opportunities and revenue to business sectors. Its demand increases throughout the years and is very much dependent on human consumption. Among the different areas of aquaculture, crustacean industry has developed rapidly in the past years due to the increase market demands of crustaceans all over the world. Prawn and shrimp industries have been considered major revenue generators, and many aquaculturists focus on cultivating shrimps and prawns as primary protein sources for consumption.

Shrimp is considered one of the most important commodities that are being traded in terms of market value. Although there is an increased production of global farmed shrimp, leading producing countries, particularly Asia, have experienced a decline in production due to shrimp diseases.

AHPND is formerly known as early mortality syndrome (EMS) for it can cause sudden, mass mortalities of up to 100% in shrimps that can be observed within 30–35 days of stocking. This disease shows greater susceptibility in *Penaeus monodon* (Asian tiger shrimp) and *L. vannamei*. The involvement of these shrimp species leaves devastating economics losses that reached about billions of dollars annually since the first outbreak in China last 2009 and consecutively affected other southeast Asian countries like Vietnam. In order to prevent AHPND disease outbreak, several countries established import bans and strengthened the import rules and condition. In 2012, the Asia Pacific Emergency Regional Consultation held a meeting in Thailand in order to address the potential risks and severity of AHPND, and this was followed by a technical workshop for the following year in Vietnam. In January 2016, AHPND was listed in the World Organization of Animal Health (OIE) so that information can easily be disseminated to its member countries to avert the disease outbreaks through updates or reports.

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