MICROBIAL AND VIROLOGICAL ANALYSIS OF *PENAEUS INDICUS* IN THE SHRIMP CULTURE FARMS OF VYPIN ISLAND

A Dissertation Submitted to St. Teresas College (Autonomous), Ernakulam in Partial Fulfilment of The Requirement for The Award of

DEGREE OF MASTER OF SCIENCE IN ZOOLOGY

SUBMITTED BY

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SANDRA MARIA

LIST OF ABBREVIATIONS

SL.NO	ABBREVIATION	EXPLANATION
1.	Eh	Redox potential
2.	pH	Potential Hydrogen
3.	WSSV	White Spot Syndrome Virus
4.	YHV	Yellow Head Virus
5.	TSV	Taura Syndrome Virus
6.	IHHNV	Infectious Hypodermal & Hematopoietic Necrosis
7.	HPV	Hepatopancreatic Parvovirus
8.	EMS	Early Mortality Syndrome
9.	IMNV	Infectious Myonecrosis Virus
10	Ha	Hectare
11.	M	Metre
12.	Mm	Millimetre
13.	ºC	Degree Celsius
14.	Mg	Milligram
15.	%	Percentage
16.	WQI	Water quality index
17.	CCME	The Canadian Council of Ministers of the Environment
18.	Ml	Millilitre

19.	µg	Microgram
20.	G	Gram
21.	CFU	Colony forming unit
22.	TPC	Total plate count
23.	$AgNO_3$	Silver Nitrate
24.	HCl	Hydrochloric acid
25.	BOD	Biochemical Oxygen Demand
26.	H_2SO_4	Sulphuric acid
27.	EDTA	Ethylenediaminetetraacetic acid
28	EBT	Eriochrome Black T
29.	DNA	Deoxyribonucleic acid
30.	RNA	Ribonucleic acid
31.	PCR	Polymerase chain reaction
32.	dNTP	Deoxynucleoside triphosphate
33.	$MgCl_2$	Magnesium Chloride
34.	Mins	Minutes
35.	TAE	Tris-acetate-EDTA
36.	EtBr	Ethidium Bromide
37.	TCBS	Thiosulfate-Citrate-Bile Salts-Sucrose agar
38.	EMB	Eosin Methylene Blue
39.	Ppt	Parts per trillion

40.	Ppm	Parts per million
41.	Mv	Linear Momentum
42.	MSA	Mannitol Salt Agar
43.	BP	Baird-Parker Agar
44.	TC	Total Coliform
45.	EC	E. Coli
46.	FC	Fecal Coliform
47.	ICMSF	The International Commission on Microbiological Specifications for Foods

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ABSTRACT

Shrimps are the aquatic organisms which have got a great commercial importance in the economy. The current study mainly aims to investigate the presence of various microbial and virological etiologies which may badly affect the health of shrimps, as well as to determine the quality of soil and water in shrimp culturing farms and ponds. The shrimp species selected to carry out the case study on shrimp pathology was *Penaeus indicus* (Indian white prawn). It was collected from two locations of Vypin island (Valappu & Elamkunnapuzha). Water and soil quality parameters like salinity, alkalinity, temperature, Eh, pH etc., were analysed to know whether the species are prone to any stress conditions. The virology examination was conducted to check out the presence of seven different viruses like White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Taura Syndrome Virus (TSV), Infectious Hypodermal and Hematopoietic Necrosis (IHHNV), Hepatopancreatic Parvovirus (HPV), Early Mortality Syndrome (EMS) and Infectious Myonecrosis Virus (IMNV). The total viable count and microbial inspection was done to find out the occupancy of different bacteria like *E coli*, *Staphylococcus* and *Vibrios* in *Penaeus indicus* (*P. indicus*).

The analysis proved the presence of White spot syndrome virus in the shrimp samples collected from Valappu, as well as the presence of diverse bacteria like *Vibrios*, *Staphylococcus spp* and Coliforms in the samples obtained from both regions. It also gave an idea regarding the aquatic environmental parameter variations from the standard values which may have contributed to the spread of pathogens due to reduced adaptive immune nature of shrimps caused by stressed conditions. The research work enabled to get a deep knowledge regarding the numerous pathogens that are associated with shrimp aquaculture and to identify the levels of environmental parameters. With more extensive studies in shrimp pathology, we can discover more pathogenic agents and can identify their features and causes, which can help to implement the pathogen preventive measures accordingly in shrimp culturing farms.

INTRODUCTION

The shrimp aquaculture industries have undergone a great growth from small beginnings to large industries by providing a lot of people with job opportunities as well as by the rich supply of highly nutritious seafoods. In India, the shrimp aquaculture takes place in marine, brackish water and freshwater environments. An area of 1.2 million ha is used for the development of brackish water shrimp aquaculture. India has got vast natural resources suitable for culturing of shrimps. The latest estimates shows that the total brackish water area developed for this purpose rises to 1,90,000 ha, which accounts for an average of 16 per cent (Pramod *et al.*,2012). The shrimp culture farms have greatly contributed to the socio-economic development in various countries and plays a major role as an important seafood industry which have created different employment opportunities. The displacement of shrimp from their natural environments to the terrestrial ponds can lead to a major exposure towards the pathogens (Briggs,2005). The inadvertent cross species transmission of contaminating pathogens also takes place while the use of artificial feeds. A great increase in the shrimp production was noticed from the different aquaculture practices but it is also linked directly to an increased incidence of several diseases caused by microbial and viral etiologies. The diseases are the major constraints for the sustainability of shrimp production in most of the countries. The biological factors along with the pond water quality plays a key role on the spread of pathogenic organisms (Menasveta,2002).

There are different types of commercially important shrimp species found in India. They mainly include, White prawn, Flower prawn, Pink shrimp, Tiger prawn, Brown shrimp, King prawn, Marine shrimp etc. Among them Indian prawn is the most widely consumed shrimp species by the population. They are found at different regions at a depth of 2-90m by inhabiting the pond bottom. They live in brackish and marine environments and are of euryhaline nature. In the case of Flower shrimps, they are mainly seen in South East Asia and are famous for their sweet flavor. Pink prawns are species of Penaeidae family. They are fished in South America and Asia. The Tiger prawns are also highly consumed type of shrimp species and they reproduce through internal fertilization which is a process different from that of other shrimps. These shrimps are large, delicious and also provide immunity power. An iodine-rich diet is provided by brown shrimps and also have a strong flavor. King prawn and Marine prawns comes under the category of large edible prawns and they have got high demands in markets.

The penaeid species belongs to the family of marine crustaceans and comes under the suborder Dendrobranchiata. Many shrimp species like Indian prawn, Tiger prawn, White leg shrimp, which has got an economic importance comes under the Penaeidae family. Among them Indian prawns are mainly used for commercial fishery as well as farming. These species are mainly of moderate to large in size and they are fished extensively with the help of trawls, gillnets and seines. Large scale culturing of penaeid prawns is been conducted in India, the most prominent species belongs to *Penaeus indicus* (*P. indicus*) and recently *Litopenaeus vannamei*, one of the exotic species was also been introduced for culture. A great amount of foreign exchange is earned with the help of *P. indicus* shrimp species as they are considered as the most esteemed and nutritious food among the other types of seafoods. Due to this reason, these species have got high range of demands in international as well as local markets (Estante-Superio *et al.*,2022).

FIGURE 1: *Penaeus indicus*

As *Penaeus indicus* have got such a great commercial importance, it is consumed by a large number of population due to its high nutritious properties. It is also commonly known as Indian prawn, and is found mainly in Africa, Malaysia, India, China and Australia. The binomial name for Indian prawn is *Penaeus indicus* and the other common names include Tugela prawn, White prawn, Banana prawn etc.

The scientific classification of *Penaeus indicus* is as follows: -

Kingdom	: Animalia
Phylum	: Arthropoda
Subphylum	: Crustacea
Class	: Malacostraca
Order	: Decapoda
Suborder	: Dendrobranchiata
Family	: Penaeidae
Genus	: Penaeus
Species	: *P. indicus*

P. indicus is considered as a marine decapod with the estuarine juveniles. It requires sandy muds at depths of 2-90 metres and has got a life span of 18 months. After hatching, it undergoes different stages to become an adult prawn. Among the shrimp species, Indian prawn gives a contribution of 2.4% to the global fisheries and 1.2 % to the global farmed shrimp production (Hosain *et al.*,2021). The production costs may vary depending on the scale of shrimp production, the different types of culture used and also based on the number of production cycles per year.

Penaeid shrimps face a lot of problems due to the presence of several infectious agents (Jayanthi *et al.*,2018). The major epizootic diseases which have been discovered in the penaeid shrimps are mainly due to the bacterial or viral etiologies but few of the diseases have protozoan or fungal agents as their source of infection, whereas the non-infectious ones are due to the environmental extremes, nutritional imbalances and genetic factors (Lightner & Redman,1994)

More than 20 viruses infect the marine shrimps and their different life stages may be susceptible to viral infections leading to mortality, deformations and slow growth (Manivannan *et al.*,2002). The most prevalent and devastating among them is the White spot syndrome virus which was first observed on *Penaeus japonicus*. Their presence is very lethal for the shrimp populations. The Yellow head virus mainly infects *Penaeus monodon* (Lightner *et al.*,1998). YHV is highly virulent and can result in 100% mortality as soon as first signs of diseases are

noted in a shrimp. These two are considered as the main viruses infecting shrimps, apart from this there are also other types of viruses like Taura syndrome virus that infects *Litopenaeus vannamei*, Baculovirus which mainly infects the insects, Myonecrosis virus, Hepatopancreatic Parvovirus, Early mortality syndrome etc.

The bacteria's associated with the shrimps can sometimes be pathogenic or they can be opportunistic. The bacterial infections also cause severe harms to the species like mortality, necrosis, discoloration of gills, white gut, lethargia etc. Bacterial septicaemia is a microbial disease found in shrimps such as *Penaeus monodon* and *Penaeus vannamei* caused by *Vibrio paraharmolytics.* It is discovered that *V.alginolyticus, V.parahaemolyticus, Vibrio spp* are the other microbes noticed in this pathogenesis. *Salmonella* and Coliforms also take part in the development of bacterial infections. Coliforms are identified in polluted environments. Luminescent bacterial diseases are caused by *V.harveyi.* It leads to the loss of production rates and downfall of economy. *Staphylococcus* is the other major form of bacteria analyzed. Brown spot disease or shell disease is caused by *Aeromonas* and *Flavobacterium.*

A wide variety of new pathogens are discovered recently and they are mainly observed in the areas of polyculture of shrimps. Their early detection enables the farmers to prevent the huge loss in the shrimp culture industries. To limit the spread of shrimp diseases in future, it is important that the national aquatic animal health policies provide the necessary guidelines for viral and microbial eradication by implementing national aquatic animal health care strategies (Scarfe,2003).

There are several diagnostic methods to find out the pathogens that affects shrimp community. They include the direct light microscopy, histopathology, electron microscopy, enhancement and bioassay methods, application of serological methods and traditional microbiology. The shrimp pathogens cannot be diagnosed with tissue culture techniques. Later highly sensitive methods were developed for the detection of pathogens which include the DNA amplification based on polymerase chain reaction (PCR) (Xie *et al.*,2008). Major factors that should be considered by the diagnosticians to determine the cause of the disease includes host, pathogen and the environment (Lightner & Redman,1998).

The water quality parameters also contribute to the spread of pathogens and it should be regularly checked to understand whether the shrimps are in stressful conditions to prevent the loss of healthy species. The farms should contain a good pollution free water supply of fresh

as well as brackish water. Water from polluted areas contains high concentrations of suspended solids and other wastes such as effluent water from industries, urban areas and other local farms etc. and it should be avoided. The poor quality of water will destroy their immunity or defense mechanisms and they will easily get prone to infections. Live or frozen feeds present a high risk of pathogen transfer because of potentially higher doses. Polyculture practices of shrimp with other invertebrates results in same conditions.

The assessment and prediction of water quality in shrimp culture is done using signal processing techniques and they mainly helps in developing water management plans. The studies regarding the water quality are conducted based on examining the negative concentrations of compounds present in shrimp ponds that destruct the growth and reproduction of organisms (Hernandez *et al.*,2011). Nowadays, the determination of the ecosystem condition is done with the help of Inference system and the Autoregressive model, which predicts the section of environmental signals using historic information and also a set of predicted variables are been assessed. All these techniques along with the conventionalmethods like titration mainly help in estimating the water quality.

Currently there is an increasing demand for shrimps in the export market and due to this the extensive culture practices have resulted in development of semi-intensive or intensive culture systems in most parts of the country. The ponds of Vypin island are perennial as well as seasonal. Here we can notice an extensive nature with little or no management. Diseases, predators, parasites and the presence of competitors mainly cause concern in these culture systems. The prawn culture practices in aquaculture farms or ponds of Vypin island are well known. Most of the perennial ponds are on seaward side of island. The are some connections with Kochi backwaters on the eastern side through a system of canals. The harvests will take place depending on high and low tides (Nasser *et al.*,1992).

Thus, the present study was carried out to categorize the presence of different microbial and virological etiologies that will disrupt the growth of *P. indicus* shrimp species in the aquaculture farms of Vypin islands along with the checking of water and soil quality parameters, as less studies are been conducted in this area by clubbing both biological and environmental factors associated with the development of various disease conditions in shrimp species which are getting developed in culture farms rather than in marine environments.

AIM AND OBJECTIVE

AIM:

The aim of this study is to examine the presence of different microbial and virological pathogens which may affect the growth of healthy shrimps in the aquaculture farms, ponds, rivers etc of Vypin island.

OBJECTIVE:

- To identify the virus, present in *Penaeus indicus.*
- To analyse the microbial organisms found in farmed shrimps.
- To estimate the soil and water quality parameters of two different shrimp farms in Vypin area (Valappu & Elamkunnapuzha).

RELEVANCE OF THE STUDY:

The case study on shrimp pathology is very relevant as its analysis may help the shrimp farmers to adopt better management practices for disease control and sustainable farming. The risk factors identified by bacteriology, virology, water and soil quality analysis provides an understanding of the disease causation and to adopt possible risk management options for reducing the likelihood of shrimp disease outbreaks. Any types of contamination in aquaculture farms may greatly affect the livelihood of farmers, thus it is necessary to conduct a regular analysis of all the risk factors that may cause contamination. Shrimp is the most important internationally traded fishery commodity and it is highly nutritious. Thus, any infection in shrimp species may dangerously affect the health of people as well as it may result in the fall of international trade and employment opportunities.

REVIEW OF LITERATURE

WATER AND SOIL QUALITY PARAMETERS

A short term and high-density shrimp culture experimentation were carried out on the shrimp species called as *Penaeus indicus* by arranging three similar enclaves made by plastic netting and bamboo stakes. It took place in a paddy-cum-shrimp filtration field located at Narakkal, Vypin during a time period from January-April, 1979. These experiments were done in the fields having good water quality according to the Water Quality Index. A total number of 500 juveniles were filled inside the enclaves, having a length of 24-48 mm, a modal size of 36 mm and an average weight of 272 mg. Feeding the shrimps with ground-nut oil cake at the level of 5% of their body weight was done daily once. The harvesting process of cultures takes place after 4 - 12 weeks. The shrimps measured 69-96 mm and weighed 3.76 g on an average, in the first harvest and those species netted after 8 weeks possessed a length range of 75-108 mm and average weight of 5.35g which was greater than that of the initially harvested ones. The size range of shrimps in third or final harvest was 42.26 times greater than that of the primary stock. Results of the investigation, proved that *P. indicus* can be used as a modal organism for further field experiments as a species for short-term as well as high-density farming in the fields having standard parameter levels (Gopalan *et al.*,1982).

One hundred naturally infected brackish water cultured shrimp species and 10 water samples of its rearing tanks were collected for the purpose of laboratory investigations. They were made to undergo microbial and pathological analysis. The water samples were examined for total bacterial count. Certain physio-chemical analysis was also carried out. Antibiotic sensitivity test of the bacteria which was been isolated, was done in addition to experimental infection of 70 shrimps with isolated bacteria. The bacteria isolated from the samples were observed as Gram negative with a total percentage of 75.5%. Aeromonas, Pseudomonas, Vibrio, Enterobacter and Citrobacter were the isolated genera from the shrimp and water samples. Clinically, the habits noticed in the shrimps affected with bacterial infection are spiral swimming, reduction of food consumption, and the loss of normal pigmentation (Aly *et al.*,2001).

Shrimps are the aquatic organisms that normally prefers to live near the pond bottom and they are highly exposed to the conditions present at the bottom region. If the cultured shrimp are

exposed to any kind of toxic materials, it may result in causing damage and destruction of the shrimp's immune power by affecting their adaptive responses. If a disease develops from the exposure to toxic materials, then many signs are identified in the species which may include mortality, reduced feeding, slower growth, and higher sensitivity to major diseases. Aerators are used to minimize the area of sludge accumulation, chemical poising of the redox system, construction of ponds to trap sludge, stirring sediments etc. It is an eco-friendly treatment and the reuse of the sediments that is drained are means to control the conditions of the pond bottom (Avnimelech *et al.*,2003).

The shrimp culture has become a major component of aquaculture. The conditions like water and soil quality parameters greatly influence the efficiency of shrimp production. An appraisal of soil and water quality management will be provided. The analysis of organic carbon takes place after the shrimp pond gets drained for the purpose of harvest and it indicates if the concentrations of organic carbon are too low to a rate less than 0.5 or too high to a rate higher than 3 or 4. The pond bottoms are dried for 2 to 3 weeks to intensify the process of oxidation of the organic matter and other substances which got reduced. pH value of the soil and agricultural limestone applied to acidic pond bottoms should be measured. In soil which has got large amount of organic matter, fertilizers which include nitrogen will be applied at 200 to 400 kg/ha to enhance the bacterial activity. Nitrate nitrogen is very effective for treating pond bottoms as it is a nitrogen source as well as a soil oxidant (Boyd, 2003).

A huge number of deaths was recorded among cultured Penaeus indicus brood stock and also among the post-larvae collected from commercial shrimp farms on the Red Sea coast during the summer season. Two types of *Vibrio harveyi* biotypes 1 and 2 was dominantly examined and identified along with other forms of microbial species like *Pseudomonas fluorescens*, Staphylococcus *spp* and *Aeromonas hydrophila*. The frequency of the occurrence of *V. harveyi* 1, 2 and other species of bacteria like *P. fluorescens, A. hydrophila and Staphylococcus spp.* in infected samples peaked 45.3, 8.0, 82.0, 7.3 and 4.7%, respectively. The origin of diseases was due to the adverse environmental conditions. High levels of temperature, ammonia, salinityand nitrite in infected ponds and also the involvement of highly virulent *V. harveyi* biotypes are some of the factors considered as disease contributors. These factors resulted in the development of a serious course of infection and were characterized by high levels of mortality

rates and there will be no symptoms in certain cases of disease development. The recovered *V. harveyi* biotypes were sensitive to antibiotic resistance provided by chloramphenicol, oxytetracycline, enrofloxacin, oxolinic acid and nalidixic acid, while it seems greatly resistant to ampicillin, kanamycin, novobiocin, sulphonamide and gentamicin (Abdel *et al.*,2005).

The effective monitoring of all the biological, physical and chemical parameters of ponds, and inlet waters enables not only to control and understand the negative circumstances for shrimp farming, but it also avoids the damages and collapse of the environmental production process. The main objective of this study was to highlight the importance of implementing a Water Quality Index (WQI). It is considered as a tool to manage shrimp farms and their surrounding environment. In order to understand the relevant factors that causes changes in the water quality and also to prevent major disease outbreaks, same technique of WQI can be used. Water quality parameters of shrimp culture ponds and the inlet waters were monitored monthly. The study took place for a period of one year, between October, 2007 and October, 2008. Physical as well as chemical parameters (salinity, alkalinity, dissolved oxygen, hardness, temperature, pH turbidity, nitrate, phosphate, silica, ammonia, nitrogen, nitrite) and also the biological parameters like fecal coliforms, chlorophyll-a, Vibrio and microbial counts were examined. To know the dependency relationship between the variables, a correlation test of Spearman was applied. To estimate the potential use of two coastal areas for shrimp culture, the technique of Hydrological Index was used. For the comparison of the water quality parameters between the shrimp farm water supply lagoon and of two coastal environments, the procedure of Canadian Water Quality Index was taken into consideration. The results provided an idea about the case that the times of the year and water quality parameters are related with stressful environmental conditions. The changes in different variables measured like temperature, salinity, pH, alkalinity, hardness, nitrate and silica, may contribute to increased environmental stress. No difference was noticed in the water quality parameters of different study sites according to the CCME WQI. The Water Quality Index applied to the different production activities is useful to monitor water quality parameters. The WQI is an important tool for aquaculture enterprises and helps in fastest interpretation of data (Ferreira *et al.*,2011).

VIROLOGY

Six different types of viral diseases are recently known in cultured penaeid shrimps. Each of these six penaeid virus diseases like *Penaeus monodon*-type baculovirus, Baculovirus penaei, hepatopancreatic parvo-like virus, baculoviral midgut gland necrosis, hepatopancreatic parvo-like virus, infectious hypodermal and hematopoietic necrosis virus, and reo-like virus of the hepatopancreas, comprise of several number of individual strains, some of them are highly pathogenic to some shrimps, while being of little importance to others (Lightner *et al.*,1989).

A survey regarding the disease development in shrimps was undertaken with the help of histopathological tools. It took place in a cross section of prawn farming region in Kochi, during a time period from March to June, 2001. Shrimp samples were collected from 26 different farms. It constituted *Penaeus monodon, Metapenaeus dobsoni* and *Penaeus indicus*.The results identified the mortality of *Penaeus monodon* because of the mixed infection of Vibriosis as well as Monodon Baculo Virus in one of the culturing farms. The various symptoms analysed was the presence of abnormal conditions like dark coloured gills, necrosiscondition of muscles, and discoloration of the shell into brown colour. This scenario was recorded in 36% of the culture areas. The most important observation while conducting the study was the absence of WSSV in all the samples investigated. No further pathological infections were recorded in the other farms surveyed (Ambipillai & Liya,2001).

The White spot syndrome virus (WSSV) caused certain biochemical and hematologicalchanges in the hepatopancreas, muscle and hemolymph, of *Penaeus indicus*. The levels of glucose, protein, carbohydrate, amino acids, hemocyanin and fatty acids were measured in WSSV-infected shrimps as well as that of the healthy shrimps. The results observed a great increase in carbohydrate and total glucose levels. It was noted in the hemolymph region of theWSSV-infected shrimp when compared to the values identified for healthy shrimp. A reductionin muscle and also the hepatopancreas of infected shrimp was found (Yoganandhan *et al.*,2003)

An invertebrate form of nidovirus is called as Yellow head virus (YHV). It has got the capability to cause severe destruction and mortality among the shrimp species like *Penaeus monodon* and *Penaeus indicus*. This lethal condition can be prevented by the treatment of

shrimps with a type of YHV-protease dsRNA. The shrimps were injected with 10^{-6} YHV. The results showed the presence of high range of virus replication and death of the species within 2 days. A strong inhibition of YHV replication was noticed after giving an injection pf 25µg protease dsRNA. The results demonstrated that YHV associated dsRNA has given a therapeutic effect and helped in the process of curation from YHV among the infected shrimps(Tirasophon *et al.*,2007).

A great economic loss and widespread mortality across the entire shrimp farming industries in Asia was caused due to the White spot disease. It was caused by WSSV. The distribution of disease seems to be uneven, and it mainly depends on a range of environmental, socio-ecological and management factors. 233 farms were surveyed in this study conducted by Hasan *et al.*,2020. It took place in southwest Bangladesh, which was a main shrimp farming region. The results found out that, where and all the better farm monitoring practices were carried out, it had reduced the formation and spread of WSSV. The management practices like improving the quality of water, maintaining constant salinity as well as managing the exchange of water will greatly reduce the WSD spread (Hasan *et al.*,2020).

BACTERIOLOGY

The bacteria present inside the Gut will contribute to the survival and maintenance of cultured shrimps. The aim of this study was to identify the amount as well as the composition of species inside the gut region in juvenile white shrimp called as *Litopenaeus vannamei.* It was reared in two types of environments. The midguts and hindguts of the shrimps were analysed on different days for the purpose of enumeration of gram-negative, aerobic bacteria. It was done by quantifying colony-forming units using standard plating techniques. The number of Bacteria was greater in shrimps cultured in wells than that were cultured in ponds on first three days. After the third day, a sudden decrease in the bacterial count was observed in the Well shrimp. There were no significant differences in the treatments which was observed on days 6 to 10. In the Guts collected from Well shrimps consisted of *Aeromonas* and *Vibrio* and they accounted for 80–850 of the bacteria on every sampling day. In the case of the Guts collected from Pond shrimp showed a greater microbial diversity and they consisted of *Pseudomonas, Aeromonas*, and *Vibrios. Flavobacterium* were analysed in Pond shrimps on days 3 and 9, but were not

identified in the Well shrimps. Greater studies regarding the gut bacteria and shrimp interactions can help in increasing the profits as well as production of shrimps. The farmers should utilise the new technologies and buy more cost-effective feeds for the process of disease control among shrimps (Moss *et al.*,2000).

A survey regarding the disease development in shrimps was undertaken with the help of histopathological tools. It took place in a cross section of prawn farming region in Kochi, during a time period from March to June, 2001. Shrimp samples were collected from 26 different farms. It constituted *Penaeus monodon, Metapenaeus dobsoni* and *Penaeus indicus*.The results identified that the main disease condition recognised was the presence of certain abnormal conditions. The discovery of chronic inflammatory lesions took place in 42% of thesurvey conducted farms. This lesion was due to the infection of microbes. In some other farms,vibriosis and hepatopancreatic pathology was analysed (Ambipillai & Liya,2001).

A study regarding the presence of microbial organisms in the muscles of shrimps which was available locally, was analyzed on the basis of aerobic plate count. *Macrobrachium rosenbergii* and *Penaeus monodon* was the species of shrimps examined. The results proved the presence of bacteria like Salmonella-Shigella and *enterobacteriaceae*. About sixteen types of bacteria were isolated and analyzed from all the samples and it showed about 3 percentage of different bacteria categorized as Shigella sp. (12.5%), *Staphylococcus aureus* (6.25%), *Vibrio sp.* (43.75%), Salmonella sp. (25%) and *Flavobacterium sp.* (12.5%) (Yousuf *et al.,* 2008).

Shrimps which are sold in markets may be spoiled with the presence of bacteria either inside or outside. This study focussed to recognize the amount or levels of microbial organisms in shrimps which were collected from the markets of a city named Dhaka. Different categories of shrimp samples were studied. Majority of them were found to be contaminated with microbes like *Aeromonas spp., Pseudomonas spp., Klebsiella spp., Staphylococcus spp.,* and Shigella spp. Among them *Aeromonas spp., Staphylococcus spp., and Klebsiella spp.* was found in higher levels. The process of multi-drug resistance developed by majority of isolates was identified by the antibiogram studies and was observed that no activity of antimicrobes was detected (Samia *et al.*,2014).

TOTAL PLATE COUNT TEST

The vibrio bacteria easily cause diseases in the shrimp culture farms. A protein membrane from *Zoothamnium penaei* which provides high immune power was extracted to overcome this problem caused by vibrio. The aim of the study was to recognize this bacterium and to estimate the Total Plate Count before and after getting exposed to the membrane. This research was mainly undertaken to resolve the destruction of shrimps in shrimp culture by the disease. The shrimp samples were taken from original habitats. It was carried out in Lamongan, East Java. The objectives in this study were to inspect the commonly noted bacteria, their Total Plate Counts as well as their Survival Rates. The research was conducted at the Wet Laboratory in Airlangga University. The results obtained without control exhibited that the microbes found was *Vibrio sp*. It showed the colony forming units as 3 x 103 CFU/gr. The presence of microbial *vibrio sp*. was observed after it is exposed with the membrane. The TPC count was 1 × 104 CFU/gr for the 2 ppm doses and 1 x 103 CFU/gr for the 4ppm doses. The Survival Rate after six days of treatment was 79% (Marwiyah *et al.*,2019)

MATERIALS AND METHODS

STUDY AREA

The present study on "Microbial and Virological analysis of Penaeus indicus in the shrimp culture farms of Vypin island" was carried out in Vypin at two different locations called Valappu and Elamkunnapuzha. Vypin is an island located in Kochi, Kerala. Valappu is a small village in Vypin island in Ernakulam district of Kerala, India (Fig 1). Elamkunnapuzha is also a village in Ernakulam district of Kerala (Fig 2). Vypin area is famous for large number of aquaculture farms located in different regions of the island.

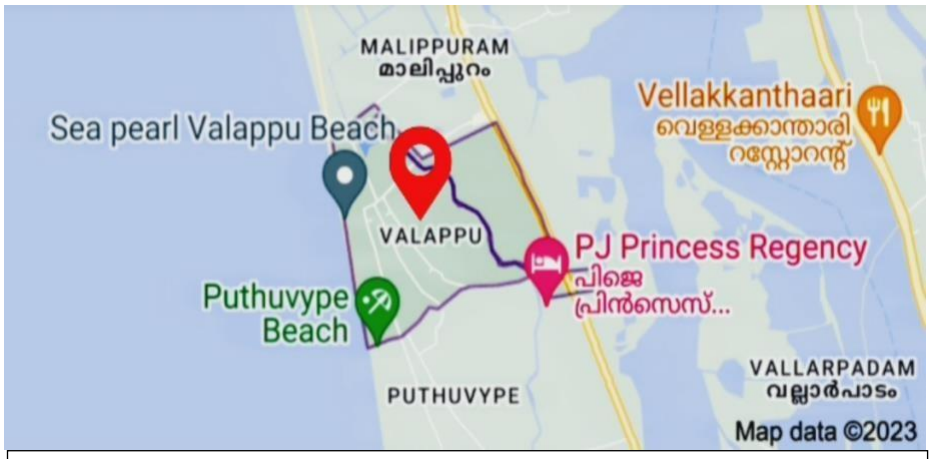

FIGURE 2: Map showing the study site Valappu in Vypin, Ernakulam

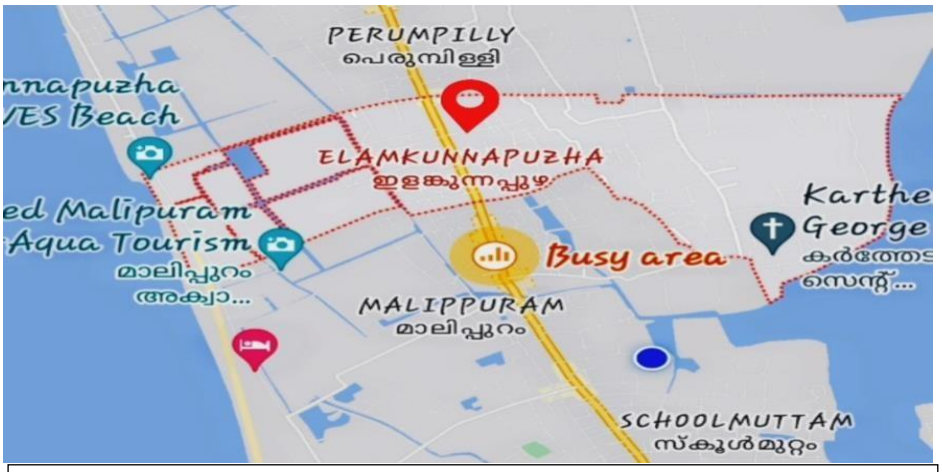

FIGURE 3: Map showing the study site Elamkunnapuzha in Vypin, Ernakulam

The risk factors associated with the shrimp species were analysed by conducting different types of tests at three different institutes. The virology tests were conducted at Cochin University of Science and Technology (CUSAT). The Total plate count tests and the estimation of soil and water quality parameters were conducted at Central Institute of Fisheries Nautical and Engineering Training (CIFNET). The bacteriology analysis was conducted at Central Marine Fisheries Research Institute (CMFRI).

COLLECTION OF SAMPLES

The shrimp species taken for the analysis of pathogens is *Penaeus indicus* (Indian white prawn). It stands as one of the major commercial shrimp species of the world. The samples were collected from the shrimp culture farms located at Valappu and Elamkunnapuzha region of Vypin island. The farmers catch the shrimp species using a fishing net and it was collected from them at suitable amounts required for different analysis. The samples for bacteriology tests were collected lively along with the water in a Ziplock cover. In the analysis of virology and total plate count tests, the samples were collected in a dead state in a Ziplock cover and was kept in freezer till the time of analysis.

PROCEDURE

I. WATER QUALITY ANALYSIS

SALINITY

The burette is filled with 0.01 N $AgNO_3$ solution. Then 10 ml of water sample is taken in a conical flask and few drops of 5% potassium chromate solution is added. Water sample is titrated against $AgNO_3$ solution. The end point is the appearance of brick red colour. The sample is titrated until the concordant values are obtained. Titration is done for a minimum of two times. The same procedure is repeated with other water sample. The salinity was found out using the below equation: -

Chlorinity = Chlorosity ÷ Density of H_2O

Salinity = $0.03 + [1.805 \times \text{Chlorinity}]$

ALKALINITY

100 ml sample is taken in a 250 ml conical flask and 2 to 3 drops of phenolphthalein indicator is added. If no colour is produced, the phenolphthalein alkalinity is considered to be zero. If pink colour develops titrate with 0.1 N HCl till it disappears or when pH attained becomes 8.3. The volume of HCl used should be noted. Then 2 to 3 drops of methyl orange is added to the same flask, and titrated till pH is brought down to 4.5 or when the orange colour is noticed as changing to pink. The alkalinity was calculated using the below equation: -

Alkalinity of sample in mg $CaCO_3$/L = [(Normality of HCl × Volume of HCl consumed ÷ Volume of sample taken)] × 50 × 1000

DISSOLVED OXYGEN

The sample is taken in a BOD bottle with utmost care to avoid any bubbling and it is filled to the neck of the bottle. Make sure that air bubbles have not been trapped under the stopper and maintain a water seal around the stopper until for the next step of analysis. Addition of 1 ml of manganous sulphate is followed by 1 ml of alkali iodide azide solution. The tip of the pipette is placed below the liquid level while adding the reagents. The stopper is placed carefully to exclude air bubbles and mix by inverting the bottle repeatedly for at least 15 minutes. Remove the stopper carefully and immediately add 1 ml of Conc.H_2SO_4, then the bottle is closed and mixed with gentle inversion until the precipitate completely dissolve. Then 50 ml of the content of the bottle is titrated with sodium thiosulphate solution using starch as indicator. The end point is observed when the blue colour changes to colourless. The starch indicator is usually added towards the end of the titration when a straw pale colour is obtained. The dissolved oxygen was estimated using the below equation: -

Oxygen content of sample = [8 × Volume of sodium thiosulphate used × Normality of sodium thiosulphate × 1000] ÷ Volume of sample.

HARDNESS

50 ml well mixed sample is taken in a conical flask and 1-2 ml buffer solution is added. To this sample, a pinch of EBT is added and titrated with standard EDTA (0.051 M) till wine red colour changes to blue. The hardness was calculated using the below equation: -

Molarity of titrant [EDTA] = 0.051M

Total hardness (mg $CaCO_3$/L) = [Volume of titrant × Molarity of titrant × 1000 ×100] ÷ Volume of sample taken.

TEMPERATURE

The estimation of temperature of water, was conducted with the help of a laboratory thermometer. For the measurement of temperature of water, a beaker was taken and filled with water sample. Here we have water samples from two areas, so two beakers were taken. Take the laboratory thermometer and observe the initial level of mercury in the thermometer and the readings are noted. Place the thermometer in the water sample and while observing the changes in the mercury level, the readings are noted down. Thermometer should be held in the water sample for 10 minutes by holding it in a straight position inside the beaker without touching the floor surface of the beaker. Then we will get a stable value and should record it.

pH

The pH of the water sample was estimated with the help of a pH meter probe. It works by inserting the pH meter probe into the solution and keeping inside it for 5 minutes. A stable value will be obtained and it is taken as the pH value of the substance. This tool enables us to quickly estimate the pH values.

II. SOIL QUALITY ANALYSIS

Eh

Soil redox potential (Eh) involves the activity of electrons and is used as a measure to estimate the soil oxygen status. The Eh value gives an idea of how a given redox reactions will occur. The activity of electrons is called as Eh. The estimation of Eh of the soil is conducted with the help of an Eh meter probe. The probe is inserted into the soil and kept for about 5 minutes. A stable value will be obtained and it will be the Eh of the substance.

TEMPERATURE

The estimation of the temperature of soil, was conducted with the help of a laboratory thermometer. For the measurement of temperature, we need to take a beaker and fill the soil in it. Here we have soil from two areas, so two beakers are taken and soil is filled in it. Take the laboratory thermometer and observe the initial level of mercury in the thermometer and the readings are noted. Place the thermometer in the soil and note the readings while observing changes in the mercury. Thermometer should be held in the soil for 10 minutes by holding it in a straight position inside the beaker without touching the floor surface of the beaker. Then a stable value is obtained and recorded.

pH

The pH of the soil was estimated with the help of a pH meter probe. It works by inserting the pH meter probe into the soil and keeping inside it for 5 minutes. A stable value will be obtained and it is taken as the pH value of the substance. It is the main tool used for quality checks of soil.

III. VIROLOGY TESTS

The virology tests are mainly conducted to check for the presence of viruses in different organisms. In this study, the tests were conducted to check the presence of seven different types of viruses that may infect *Penaeus indicus* shrimp species. The viruses include the White spot syndrome virus, Yellow head virus, Taura syndrome virus, Infectious hypodermal and hematopoietic necrosis virus, Hepatopancreatic parvovirus, Early mortality syndrome and Infectious myonecrosis virus. Table 1, shows the different methodologies for the analysis of viruses from shrimp gills.

TABLE 1: The methodology to check viruses from shrimp gills

SL.NO	PARAMETER	METHODOLOGY	SAMPLE
1.	White Spot Syndrome Virus (WSSV)	Lo *et al.*, 1996, Disease of aquatic organisms	Gills
2.	Yellow Head Virus (YHV)	Wongteersupaya *et al.*, 1997, Disease of Aquatic Organisms 31:181 - 186	Gills
3.	*Penaeusvannamei* infectious subcutaneous & hematopoietic organ necrosis/ IHHNV	PCR detection (Tang *et al.*,2000)	Gills
4.	Infectious Myonecrosis Virus (IMNV)	OIE manual, Diagnostic Test for Aquatic Animals. 2009. Chapter 2.2.3	Gills
5.	Taura Syndrome Virus	PCR based detection method	Gills
6.	Hepatopancreatic Parvovirus disease (HPV)	PCR based detection method (Khumsirichart *et al.*, 1999)	Gills
7.	EMS	PCR based detection method	Gills

The virus detection was done using the following procedures: -

A) DNA ISOLATION

DNA isolation was done by DNA – Xpress kit method digestion buffer

The digestion buffer used in this procedure is STE Buffer (1 ml for one sample)

5M NaCl - 20 µl

1M Tris HCl - 200 µl

0.5 M EDTA - 100 µl

Milli Q - 680 µl

The tissue with digestion buffer (200 µl) was homogenized. The sample + STE Buffer 800 µl + 50 µl SDS + 30 µl proteinase K was taken in a new ependroff tube. It is mixed well and kept in water bath in 60º C for 1 hour and at every 15 minutes invert the tube. Centrifuge them at 10000 rpm for about 10 minutes. About 500 µl of supernatant is taken and to that mix add equal amount of DNA xpress. Centrifuge at 10 minutes at 10000 rpm. By using cut tip 500 µl supernatant is taken in a new 1.5ml MCT. Added 1 ml 100 % chilled ethanol to it. Keep it at -20º C for 10 minutes. Centrifuge at 12000 rpm at 10 minutes (Transparent pellet formation will occur). Decant the ethanol and 500 µl of 75% ethanol is added to it. Centrifuge and decant again. Dry the precipitate and added 20 µl milli Q. Dissolve and store in -20º C.

B) RNA ISOLATION

From shrimp gills, 50-100mg tissue per ml of RNAiso Plus reagent was extracted. DEPC treated needles were dipped in 10% cold sodium citrate made in DEPC treated water. The homogenizer was placed with RNAiso Plus reagent in ice. The gills were smashed with the anticoagulant dipped needles into homogenizer. After it is homogenized thoroughly with the pestle, add more RNAiso Plus reagent proportionate to the amount of sample to retain the pink colour. Then it is transferred into fresh 2ml MCTs. Keep the samples for about 5 minutes at the room temperature. Addition of 0.2 ml of chloroform (free of isoamyl alcohol/additives) /ml

of RNAiso Plus reagent takes place. The samples were covered and shaken continuously for 15 seconds. It was allowed to stand for 15 minutes at room temperature (max:30 mins). Centrifuge the resulting mixture at 12000g for 15 minutes at 4º C. Three phases are developed after centrifugation. Red organic- protein containing phase, Interphase- DNA containing phase and Colourless upper aqueous- RNA containing phase. The colourless aqueous phase was transferred into fresh 1.5 ml MCTs. Interphase and Organic phase were stored at 4º C for DNA and protein isolation. Addition of 0.5 ml of isopropanol/ml of RNAiso Plus reagent, followed by turning of MCTs 5/6 times. Samples were allowed to stand for 10 minutes at room temperature. Centrifuge at 12,000 g for 10 minutes at 4º C. On side and bottom of the tube a precipitate is formed by RNA pellets. Decant the supernatant immediately after centrifugation. RNA pellet was washed by adding 1 ml of 75% ethanol/ml of TRI reagent. Vortex samples and pool to a single tube. Centrifuge at 12,000g for 5 minutes at 4ºC. The supernatant was removed and repeat the steps and the final centrifugation at 12,000g for 10 minutes at 4º C. Then the supernatant is removed and the tubes are kept in tissue paper under table lamp light for drying till no ethanol drop is left (10-15 minutes, max 30 minutes). Addition of 20 µl of DEPC treated water to the MCTs and they are thoroughly vortexed. It was spinned in mini centrifuge and was incubated at 55.5º C for 10 minutes. Then finally place them in ice.

DNase TREATMENT

DNase Buffer (10X) = 1.75 µl

Template RNA = 17.5 µl

DNase enzyme = 0. 2 µl

DEPC H_2O = 0.66 µl

37 degree Celsius = 10 mins

75 degree Celsius = 10 mins

cDNA SYNTHESIS

RNase Inhibitor = 0.5 µl

dNTPs (10mM) = 0.2 µl

Oligo dt(10pmol/µl) = 4.0 µl

RT buffer 1 = 2.5 µl

$MgCl_2$ (25mM) = 1.6 µl

RT enzyme = 1.0 µl

Vortex and spin.

QUANTIFICATION OF RNA

The quantification of RNA was carried out using an instrument called as photonanometer. There are different steps for the functioning of this instrument. First, the instrument is switched on and the option of Nucleic acids is selected. Press the option called as Blank and note the readings. After this step, the front lid of the instrument was opened in order to apply the RNA sample. Then the sample option was clicked and the readings were noted. Repeated the same steps for getting the values of RNA quantification with the second sample.

C) POLYMERASE CHAIN REACTION (PCR)

PCR was conducted to find out the presence of seven different viruses in *Penaeus indicus*. The conditions in the PCR will be different for the seven types of viruses. Table 2, shows the PCR conditions of those seven viruses.

TABLE 2: PCR conditions of Viruses

Sl No.	Viruses	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Hold	Cycle
1.	WSSV	95º C 5 mins	95º C 30 sec	55º C 30 sec	72º C 60 sec	72º C 10 mins	25º C	34
2.	YHV	94º C 2 mins	94º C 30 sec	58º C 30 sec	72º C 30 sec	72º C 10 mins	25º C	39
3.	TSV	94º C 2 mins	94º C 45 sec	60º C 45 sec	60º C 60 sec	60º C 7 mins	25 ºC	39
4.	IHHNV	95º C 3 mins	95º C 30 sec	55º C 30 sec	72º C 60 sec	72º C 7 mins	25º C	34
5.	HPV	95º C 5 mins	95º C 30 sec	55º C 30 sec	72º C 60 sec	72º C 10 mins	25º C	34
6.	EMS	95º C 5 mins	95º C 30 sec	60º C 30 sec	72º C 60 sec	72º C 10 mins	25º C	34
7.	IMV	95º C 2-3 mins	94º C 45 sec	68º C 45 sec	72º C 60 sec	72º C 5 mins	25º C	39

There are different steps for the functioning of PCR.

The instrument was switched on and 'Files' option was selected. Then Select the 'Load' option and click the desired virus name or program. It was edited with new temperature andconditions. When we click Exit, a question appears asking whether to save or cancel the settings. Click Yes and press the 'Enter' option. The sample was loaded inside the instrument and then a question appears asking whether the quantity is 0.2. click Yes and press the Enter option. Then the value of the sample should be entered. Click 10 µl and press Enter and then 'Start the Program'. The 'Opt' button was clicked to find the time at which the program will end.

D) AGAROSE GEL ELECTROPHORESIS

The composition of agarose gel preparation is as follows: -

Gel preparation – 40 ml

1.Agarose – 0.4 g

2. TAE (1X) – 0.6 ml (50X)

3. EtBr – 1 µl

4. Distilled water – 40 ml

Agarose gel was prepared by combining 0.4 g agarose in 1X TAE buffer and heating in a microwave until the agarose is dissolved (1min). After adding 1µl ethidium bromide (EtBr), swirl to mix, and the gel was poured onto a taped plate with casting combs in place. Agarose should be poured without introducing air bubble from the comb end to the other. It was placed 20 to 30 minutes for solidification. Remove the gel casting combs and tape, then place the gel in an electrophoresis unit. 1X TAE buffer was added to the reservoirs until it covers the agarose gel. Added one – tenth volume of 10X agarose gel loading dye to each DNA sample, and was mixed in a paraffin strip, and loaded into the wells. The lid of the electrophoresis unit was placed carefully. The power pack were switched on and apply 110V. After the required

separation has been achieved, the power was switched off and the gel was taken out from the apparatus. The lower parts were wiped with tissue paper. The DNA fragments was then visualized on a long wave UV light in Gel Doc.

E) GEL DOC

A gel doc, is a gel documentation system or gel image system, widely used in molecular biology laboratories for forming the images of nucleic acids and protein which is present within the agarose gels. These gels are mostly stained with ethidium bromide. The main components of Gel doc include the ultraviolet light transilluminator, a darkroom to defend external light sources and also to protect from UV radiations, and a CCD camera for capturing images. The fragments of DNA are referred to as 'bands' due to their presence on the gel. Here, Gel doc is used for DNA band visualization. There are certain steps for the working of this software.

The option called 'Image lab' app was selected and from that the 'Files' option was clicked. The option called as 'New protocol' was selected. In the applications, select the nucleic acid gel option and click on ethidium bromide. Then, unmark the option 'highlight saturated pixels. Select the position gel option and click OK. Check whether the position of the gel is correct and press Run protocol option. The DNA bands will be visualized.

V.BACTERIOLOGY TESTS

The bacteriology tests were conducted to find out the presence of bacteria like *E. coli, Staphylococcus* and *Vibrio*.

The *Penaeus indicus* shrimp species were pooled and proceeded for microbial analysis. The whole organism along with gut homogenized using sterile normal saline. Subsequently, 10-fold dilutions of homogenate were prepared and each dilution was spread onto nutrient agar, Thiosulphate- Citrate- Bile Salts- Sucrose Agar (TCBS agar), MacConkey agar, Eosin Methylene Blue (EMB), Baird – Parker agar and Mannitol salt agar. All the plates except nutrient agar and TCBS were incubated at 37º C for 72 hours. Nutrient agar and TCBS were incubated at 30º C for 3 days. The colonies characteristic of targeted microbes was only taken into consideration during enumeration.

IV.TOTAL PLATE COUNT TESTS (TPC)

For spread plate method (Surface plate method), Agar plates were poured and dried in advance. About 11.7g of plate count agar was melted in a water bath, one flask of the medium was cooled to 45ºC and poured into the petri dishes. It was then allowed to set. The surface of the medium was dried either in a 56ºC incubator or in a laminar flow chamber for about 45 min., and after that the plates were cooled to the room temperature.

SAMPLING

The glass mortar (interior) and the pestle were sterilised by smearing with alcohol and flaming. It was rinsed with the distilled water. 1g of the whole-body was aseptically cut out from the shrimp sample into a sample dish. Macerated with 9 ml distilled water in a sterile glass mortar (10^{-1} dilution). 1 ml of the supernatant was then pipetted to 9 ml distilled water and mixed well (10^{-2} dilution).

FOR SPREAD PLATING

The petri dishes in which the plate count agar is already poured and allowed to set was taken and labelled appropriately. 1 ml each of the 10^{-1} and 10^{-2} dilutions on the surface of the respective agar plates was inoculated. With the help of a highly sterilised bent glass rod spread the inoculum on the surface of each petri plate uniformly. The glass rod must be sterilized by dipping it in alcohol and then flaming, in between spreading operations of each plate.

After about 30 min, incubation of the plates at 37ºC (or room temperature) takes place for about 48 hours. After 48 hours of incubation, the colonies were developed in each plate are they were counted using a Quebec colony counter. The colony counts of duplicate plates should agree within 10% limit and the counts between decimal dilutions should agree decimally.

TPC/g sample is calculated using the relation: -

TPC/g sample = Average count × (1 ÷ dilution factor).

RESULTS

I.WATER QUALITY PARAMETERS

SALINITY

Salinity of water sample from Valappu was 20 ppt while that from Elamkunnapuzha was 15 ppt.

ALKALINITY

Alkalinity of water sample from Valappu was 175 ppm while that from Elamkunnapuzha was 170 ppm.

DISSOLVED OXYGEN

Dissolved oxygen of water sample from Valappu was 4 ppm while that from Elamkunnapuzha was 10.8 ppm.

HARDNESS

Hardness of water sample from Valappu was 3346 while that from Elamkunnapuzha was 2642.

pH VALUE

pH of water sample from Valappu was 9 while that from Elamkunnapuzha was 7.5.

TEMPERATURE

Temperature of water sample from Valappu was 31ºC while that from Elamkunnapuzha was 30ºC.

Table 3, shows the tabular representation of the values obtained for water quality analysis.

II. SOIL QUALITY PARAMETERS

TEMPERATURE

Temperature of soil sample from Valappu was 30.5ºC while that from Elamkunnapuzha was 31ºC.

pH VALUE

pH of soil sample from Valappu was 7.6 while that from Elamkunnapuzha was 8.4.

Eh VALUE

Eh of soil sample from Valappu was -473 mV while that from Elamkunnapuzha was -295 mV.

Table 4, shows the tabular representation of the values obtained for soil quality analysis.

WATER QUALITY PARAMETERS

TABLE 3: Values obtained for water quality parameters

SL.NO	WATER QUALITY PARAMETERS	SAMPLE FROM VALAPPU	SAMPLE FROM ELAMKUNNAPUZHA	STANDARD PARAMETERS
1.	SALINITY	20 ppt	15 ppt	10 – 20
2.	ALKALINITY	175 ppm	170 ppm	75 – 150
3.	DISSOLVED OXYGEN	4 ppm	10.8 ppm	More than 6
4.	TOTAL HARDNESS	3346 ppm	2642 ppm	Depends on salinity. If 10 = 3000 ppm
5.	TEMPERATURE	31ºC	30ºC	25ºC - 35ºC
6.	Ph	9	7.5	7 – 8

SOIL QUALITY PARAMETERS

TABLE 4: Values obtained for soil quality parameters

SL.NO	SOIL QUALITY PARAMETERS	SAMPLE FROM VALAPPU	SAMPLE FROM ELAMKUNNAPUZHA	STANDARD PARAMETERS
1.	TEMPERATURE	30.5ºC	31ºC	25ºC - 35ºC
2.	Ph	7.6	8.4	7 – 8
3.	Eh	-473	-295	-300 and +900 mV

From water quality assessment, it is clear that there is a slight difference in water quality parameters like alkalinity, dissolved oxygen, hardness and pH from the standard value of parameters. It is also found that the Eh value of soil parameter also shows a variation from the normal values. It indicates that the shrimp species growing in these locations undergo certain kinds of stress conditions. The changes are greatly noticed in the sample collected from Valappu region. As they are in such a stressed condition there are great chances for the shrimp species to get affected with several pathogenic infections due their reduced adaptive immune complex. There will be a great decline in the quantity of shrimp species cultured in the farms as so many of them will reach the verge of death. Thus, it is very important for the farmers to conduct the water and soil quality parameter checks at regular intervals.

III. VIROLOGY RESULTS

The virology tests are mainly conducted to check for the presence of viruses in different organisms. In the current study, the presence of seven different types of viruses that may infect *Penaeus indicus* shrimp species were checked. The viruses include the White spot syndrome virus, Yellow head virus, Taura syndrome virus, Infectious hypodermal and hematopoietic necrosis, Hepatopancreatic parvovirus, Early mortality syndrome and Infectious myonecrosis virus. The results of virology tests are as follows: -

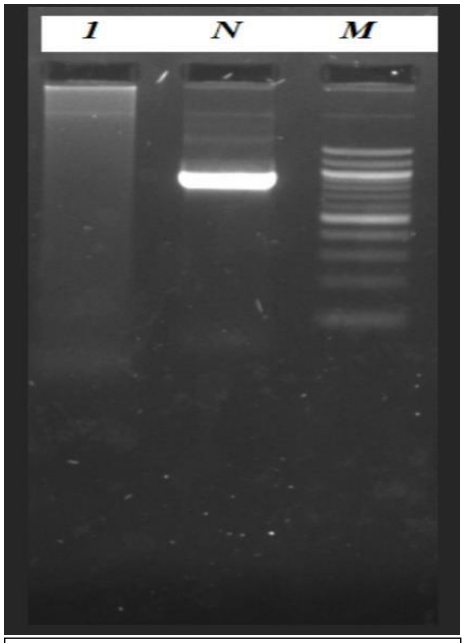

FIGURE 4: Virology test shows positive result for the presence of WSSV Virus

FIGURE 5: Virology test shows negative result for the presence of Viruses

White Spot Syndrome Virus (WSSV)

Primer sequence (set 1):
WSSVF15'-ACTACTAACTTCAGCCTATCTAG-3'
WSSVR15'-TAATGCGGGTGTAATGTTCTTACGA-3'
Expected Product size: 1447bp

Primer sequence (set 2):
WSSVF2 5'-GTAACTGCCCCTTCCATCTCCA-3'
WSSVR2 5'-TACGGCAGCTGCTGCACCTTGT-3'
Expected product- size: 941bp

RESULT: - Visible band was observed with 941 bp in the gel and expected as positive against WSSV virus.

The virology tests revealed that, out of seven viruses checked, one was found positive in *Penaeus indicus* shrimp species. From the pictorial representation, it is evident that the result is positive for the presence of White Spot Syndrome Virus, as a visible band was observed in accordance with 941 base pairs in the gel. Many symptoms will be noticed in the shrimps affected with WSSV but in this case, the shrimp species collected had no such symptoms. Studies shows that the symptoms may not be greatly visible during the initial periods of WSSV attacks.

TABLE 5: Results of Virology tests

SL.NO	PARAMETER	RESULT
1.	White Spot Syndrome Virus (WSSV)	**POSITIVE**
2.	Yellow Head Virus (YHV)	Negative
3.	*Penaeusvannamei* infectious subcutaneous & hematopoietic organ necrosis/ IHHNV	Negative
4.	Infectious Myonecrosis Virus (IMNV)	Negative
5.	Taura Syndrome Virus (TSV)	Negative
6.	Hepatopancreatic Parvovirus disease (HPV)	Negative
7.	Early Mortality Syndrome (EMS)	Negative

IV. BACTERIOLOGY RESULTS

The bacteriology tests were conducted to examine the presence of bacteria like *E. coli, Staphylococcus* and *Vibrio*. Different medias were used to test the presence of various bacteria, from the specimen collected from Valappu and Elamkunnapuzha. The results of bacteriology tests are as follows: -

TABLE 6: Results of Bacteriology tests

Media/ Test	**Microbes Targeted**	**Valappu (CFU/g)**	**Elamkunnapuzha (CFU/g)**
TCBS Agar	Presumptive vibrios	1×10^2	5.5×10^2
MacConkey Agar	Presumptive coliforms	1.5×10^2	0
EMB Agar	Presumptive *E. coli*	0	0
MSA	Presumptive mannitol fermenting *Staphylococcus spp.*	0	2×10^2
BP Agar	Presumptive *Staphylococcus aureus*	0	1×10^2

The bacteriology tests revealed that, both the specimens collected from two areas of Vypin were prone to certain types of bacterial infections. In the case of specimen collected from Valappu region, it showed the presence of bacteria like Vibrios and Coliforms but other bacteria like *E coli* and *Staphylococcus* were absent. The colony-forming unit per gram of specimen when the microbe targeted was *Vibrio* were 1×10^2 CFU/g and when the microbe targeted was Coliforms, it was 1.5×10^2 CFU/g. When it comes to the specimen collected from Elamkunnapuzha region, there noticed the presence of bacteria like *Vibrios* at a rate of 5.5×10^2 CFU/g, *Staphylococcus spp* and *Staphylococcus aureus* forming a colony unit of 2×10^2 CFU/g and 1×10^2 CFU/g. *E coli* and Coliforms were found to be absent. It is evident from the data that

E coli was absent in both the areas. So, the shrimp farms were found mostly to be infected with bacteria like Coliforms, *Vibrios* and *Staphylococcus spp*. If control measures are not properly practiced, the spread of bacterial colonies may take place and result in mortality of shrimp species.

V. TOTAL PLATE COUNT TEST

Total plate count tests were conducted in Gut as well as in the Whole body of *Penaeus indicus* species.

TABLE 7: Results of Total Plate Count Tests

Media/ Test	**Microbes Targeted**	**Valappu (CFU/g)**	**Elamkunnapuzha (CFU/g)**
Nutrient Agar	Total viable count in the Gut of *Penaeus indicus*	9.5×10^2	7.5×10^2
Plate count Agar	Total viable count in the Whole body of *Penaeus indicus*	42×10^3	31×10^3

The Total Plate Count tests proved that the shrimp species have got a great presence of various bacteria and the Whole-body analysis showed a huge colony-forming unit value when compared with that of the Gut region. The total viable count in the Gut of *Penaeus indicus* from the specimen collected from Valappu were 9.5×10^2 CFU/g and from Elamkunnapuzha were 7.5×10^2 CFU/g. In the case of the total viable count in the Whole body of *Penaeus indicus*it was 42×10^3 CFU/g in Valappu and 31×10^3 CFU/g in Elamkunnapuzha.

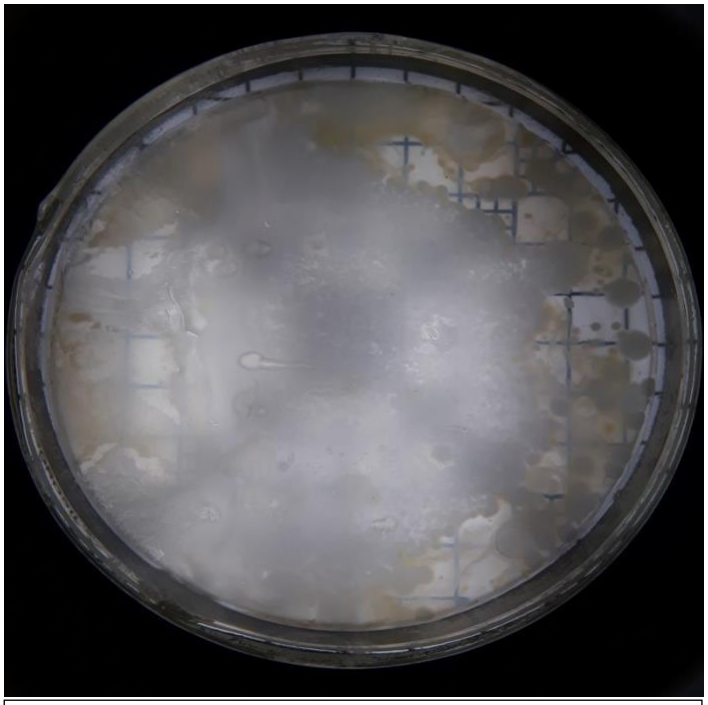

Figure 6: Total plate count test shows the presence of bacterial colonies from the sample tested from Valappu (10^{-3} dilution)

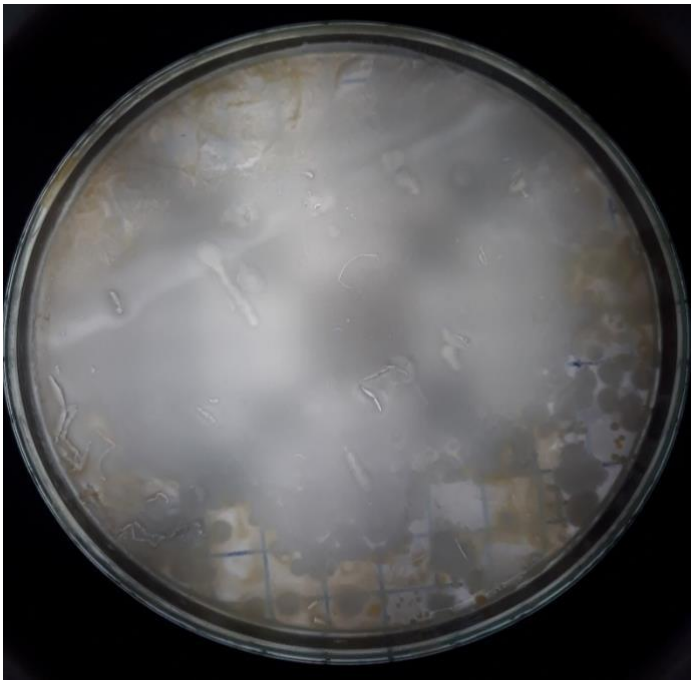

Figure 7: Total plate count test shows the presence of bacterial colonies from the sample tested from Elamkunnapuzha (10^{-3} dilution)

DISCUSSION

The shrimps are one among the most esteemed food and economically important aquatic organisms of India. It enables to earn a huge amount of foreign exchange. It was noticed that these organisms were affected by different types of pathogens which have the ability to lead to mortality of shrimp species and this may create a huge loss in international trades. So, in order to find out which pathogens mainly contributes to the shrimp disease outbreaks, we conducted microbial and virological investigation along with the detection of water and soil quality parameters of shrimp farms.

The present study examined the risk factors affecting the shrimps in their culture systems, which was carried out for a duration of 1 month. *P. indicus (*Indian white prawn) was taken as the shrimp species for analysis and the specimen was collected from two shrimp culture farms of Vypin island (Valappu & Elamkunnapuzha). The major tests that were conducted for this purpose includes virology, bacteriology, TPC, water and soil quality parameters. The virology tests were conducted for seven different viruses in *P. indicus*. The presence of White spot syndrome virus was detected in one of the two samples that was taken for analysis. It was the sample from Valappu region and the farmers of this region mentioned that there is already certain presence of this virus and they are taking necessary precautions for its control. The results of the TPC and bacteriology tests revealed the presence of bacteria like Coliforms and *Vibrios* in the sample collected from Valappu and the presence of bacteria namely *Staphylococcus* and *Vibrios* in the sample obtained from Elamkunnapuzha. The water and soil quality parameters assessed, from two areas had given the values which showed some variations in its levels when compared with the standard parameter levels. The changes were observed in alkalinity, dissolved oxygen, hardness and pH ranges of water quality and slight differences were also detected in Eh value of soil quality parameter.

The previous study conducted by Carbajal *et al.*, (2013), was based on the importance of maintaining the water quality parameters in shrimp culture systems. Water quality assessment is the main activity for controlling harmful crisis in aquaculture farms. To develop a new strategy of Water Quality Index was the main objective of their research work which greatly

focused on inspection and controlling of shrimp farms by detecting poor quality of water and preventing ill effects in the ecosystem. Usually, various water quality assessments are conducted and measured in a shrimp farm during a farming period. The classification of parameters takes place according to their pessimistic effects in the ecosystem and the respective limits which are considered as the standard values are also defined. The Water Quality Index proposed by the study assigns a precedence level to each water parameter through different analytical process, which allows a precise assessment of the water quality. This study has got a correlation with the current study as all the parameters analyzed in shrimp pathology is included in the Water Quality Index and the reports that was obtained related with water quality parameters were checked using the standard values involved in Water Quality Index. Using this policy, helped in converting the composite water quality determination data into information that is comprehensible and accessible by the public (Carbajal *et al.,* 2013).

According to Yoganandhan *et al.,* (2003), they carried out PCR at different time intervals to detect white spot syndrome virus (WSSV) in shrimp samples obtained from several culture locations. The PCR analysis showed that hemolymph was positive for WSSV, and in the present case the analysis of seven different viruses were checked by the same PCR technique by collecting samples at varied time intervals from culture farms. The study showed that the gills were positive for White spot syndrome virus. So, it is evident that gills of shrimps can also be taken as a sample apart from hemolymph region which is mainly mentioned in most studies to check the viral presence in shrimps (Yoganandhan *et al.,* 2003).

White Spot Disease (WSD) caused by WSSV is a great threat for shrimps and till date no efficacious treatment is available against the disease. In the current research, the major cause discovered as the factor of virus infection in shrimps was their sudden displacement from natural environment to terrestrial farms and the presence of stressful environmental conditions which disrupted the adaptive complex immune system of shrimps which made it easily prone to viral attacks due to less immune power. Moreover particulars regarding the mechanism of ingress of White Spot Syndrome Virus (WSSV) into shrimps were studied by Verma *et al.,* (2017), by describing the protein interactions occurring between WSSV and shrimp during viral entry. Various shrimp proteins play a key role in the entry mechanism of WSSV virus and

it was found that protein VP26 and VP466 are the major structural proteins that enables the WSSV interaction and entry into the species (Verma *et al.*, 2017).

In accordance with Dallal *et al.*, (2015), *Staphylococcus aureus* (*S. aureus*) is one of the most common causes of shrimp diseases. The principal focus of this study was to collate the spread and contamination of *S. aureus* in farmed as well as marine shrimps in Tehran fishery center. The results of this investigation exhibited that 20% of farmed shrimps and 30% of marine shrimps were contaminated with *S. aureus*. Due to the high contamination rate of shrimps by *S. aureus,* increased heed should be given during its refining and manufacturing processes. This major study has got a slight relation with my research as there is the presence of *S. aureus*in the sample collected from the shrimp culture farm of Elamkunnapuzha region and their presence was identified using conventional methods. The colony forming unit was identified as 2×10^2 CFU/g in the case of presumptive *Staphylococcus spp* and it was 1×10^2 CFU/g in presumptive *S. aureus* (Dallal *et al.,* 2015).

As we visited the sites for sample collection, the farmers mentioned that they added different probiotics to control bacterial spread. In order to control *Vibrio,* they mainly used a probiotic called as *Anti-vibrio.* In the previous studies carried out by Moriarty, (1998), a catastrophe has made an appearance in the prawn processing industry in many locations with the outset of disease, with *Vibrio* spp. being the important factor for its cause. Here, the probiotic bacteria added to control the *Vibrio* was certain selected strains of *Bacillus* and it is conveyed by comparing the culturing farms in Indonesia using the identical water sources, which constituted luminous *Vibrio* strains. The farms that did not use the cultures of *Bacillus* as probiotics showed a complete failure in every pond taken for analysis by getting highly contaminated with luminous *Vibrio* and it resulted in mortality of shrimps before 80 days of culture. In contrast, a farm using the probiotics was culturing shrimps for about 160 days without calamities, by using strains of *Bacillus* as probiotics at high rates of about 1×10^4/ml to 1×10^5/ml. The composition of microbes was different in the pond water of the two culture farms and signify that it is possible to change the microbial species configuration and improvethe shrimp production in large water bodies (Moriarty, 1998).

In the studies conducted by Rana & Domingo, (2017), a group of coliform bacteria is widely used as the pollution indicator which mainly exhibits the presence of pathogenic microbes that is greatly linked to faecal contamination. It possesses greater risks associated with the health of shrimp community. This study focusses to institute the baseline information on the coliform contamination in aquaculture farms. The water samples and important aquaculture products were collected two times during each season from respective aquafarms in the coastal provinces of the bay and were estimated for total coliform (TC), E. coli (EC) and fecal coliform (FC) by using the conventional methods. TC, EC, and FC in water were found in lower rates during the wet season, while it was found in higher rates in the dry season. The current investigation proved the presence of contamination in shrimps from one sample site called Valappu as they showed 1.5×10^2 CFU/g of presumptive Coliforms. It may be one of the serious contributors of shrimp disease outbreaks as shrimps lose their immune responses in polluted environments. The high rate of pollution may be caused due to the presence of high concentrations of suspended solid materials and other effluents from industries, urban areas etc. The tests were conducted by conventional methods during the wet season, so that the number of Coliforms was in lower rates as per the studies of Rana & Domingo, (2017).

The microbial quality of Indian white shrimp (*Peneaus indicus*) collected from Bagerhat, Khulna and Satkhira of Southwest Bangladesh was assessed by Nilla *et al.,* (2012). The parameters showed variations with different sources and the quality was identified to be poor for Satkhira shrimp samples. The Total bacterial count of all the samples was beyond the acceptable limit of 10×10^2 CFU/g recommended by ICMSF. In the case of total coliform and E. coli density, no substantial changes were observed among the different shrimp samples from different districts. While comparing these results with the present analysis, it was found that the total plate count level in shrimp samples was below the acceptable limit in gut and above the acceptable limit in the whole body. In gut, the colony forming unit were found as 9.5×10^2 CFU/g and 7.5×10^2 CFU/g in Valappu and Elamkunnapuzha. In whole body, it was 42×10^3 CFU/g and 31×10^3 CFU/g respectively (Nilla *et al.,* 2012).

As per the studies conducted by Scarfe, (2003), several effective disease management strategies have been identified to prevent the spread of pathogens and for the healthy production and growth of shrimp species in the aquaculture farms. As the shrimp species are invertebrates,

they lack the vertebrate adaptive immune responses which provides them with a mechanism of natural form of protection. The principles like avoidance of environmental conditions that cause stress and pathogen exclusion are taken as the important strategies for health management in shrimp aquaculture. The pathogen exclusion requires a great attention to shrimpproduction cycles that starts from spawning to harvest period. To limit the spread of shrimp diseases in future, it is important that the national aquatic animal health policies provide the necessary guidelines for viral and microbial eradication by implementing national aquatic animal health care strategies. The current study also focusses on this principle of pathogen exclusion by controlling the environmental parameters by bringing them into normal levels byconducting regular examinations (Scarfe, 2003).

The solutions to the problems associated with shrimp aquaculture may mainly lie in the development of new technologies. The conventional vaccination method is not possible due to the lack of adaptive complex immune system according to Robalino *et al.,* (2007). The previous studies show that shrimps are developing a wide range of newly evolved mechanisms to respond to viruses and microbial organisms. RNA interference has been shown to have a great potential for application in shrimp and it is a major antiviral strategy. The current study involves the injection of dsRNA or short interfering RNA which can effectively block WSSV,TSV and YHV infection and their injection is nowadays common and widely used for the disease prevention in shrimps (Robalino *et al.,* 2007).

The present study identified that the farms should contain a good pollution free water supply of fresh as well as brackish water. Water from polluted areas contains high concentrations of suspended solids and other wastes such as effluent water from industries, urban areas and other local farms etc. and it should be avoided. The presence of certain types of bacteria like Coliforms, *E. coli* can also indicate that the water is polluted as these bacteria can live only in such a highly polluted environment. In the previous study conducted by Rajasegar, (2003), he explained that the shrimps will be spending most of their time in soil areas or the pond bottom during their culture period. While choosing the sites, the areas with sandy or silty soil should be avoided that may lead to the erosion, easy infiltration of the wastes into the soil areas and seepage of water. Other types of soils like mangrove or acid sulfate soil are not used for shrimp pond culture as they have got high organic matter contents. We can also notice an acidic nature

in those soil which require a high-water exchange rate as well as low stocking density. Thus, a pollution free water supply along with the measures developed to eradicate the pathogen spread will prevent the formation of infectious diseases in shrimp culture farms (Rajasegar, 2003).

CONCLUSION

The case study on shrimp pathology was conducted on the shrimp species called as *Penaeus indicus* (Indian prawn) as it is one of the major commercial prawn species of the world. The sample was collected from Valappu and Elamkunnapuzha regions of Vypin island. It was found that the shrimps face a lot of risk factors that will affect their growth as well as the livelihood of farmers. It can also severely affect the health of people as they consume them due to their nutritious properties. The risk factors affecting *P. indicus* was examined by conductingvirology and bacteriology tests. The presence of seven different viruses were tested and it wasfound that the sample showed positive results for White spot syndrome virus and all other six viruses were shown as negative. The bacteriology and total plate count tests were alsoconducted in order to find out their presence and total number of bacteria in the shrimp species.The tests showed positive results for the presence of *Staphylococcus, Vibrios* and Coliforms.

E. coli was absent in both the areas. The total plate count tests proved that the microbial load was more in the sample obtained from Valappu when compared with that of Elamkunnapuzha. Apart from this water and soil quality analysis were also experimented to check whether the shrimps are under stressful conditions. Variations of values from standard parameter values proved that the organism is susceptible to some kinds of pressurized surroundings. This study helps the farmers to take necessary precautions against the development of pathogens in the shrimp culture systems. The risk factors identified by the study provides an understanding of disease causation and to adopt possible risk management options for reducing the likelihood of shrimp disease outbreaks. Thus, through this investigation, a lot of information regarding the pathogens that infect shrimps are understood and more detailed analysis can be undertaken if an extensive study regarding this topic is conducted.

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