BACTERIOLOGICAL AND VIROLOGICAL ASSESSMENT OF CULTURED *PENAEUS MONODON* IN VYPIN AND CHELLANAM AREAS

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SUBMITTED BY

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LIST OF ABBREVIATIONS

SL NO	ABBREVIATION	EXPLANATION
1	pH	Potential Hydrogen
2	Eh	Redox potential
3	YHV	Yellow Head Virus
4	IHHNV	Infectious Hypodermal & Hematopoietic Necrosis
5	IMNV	Infectious myonecrosis virus
6	TSV	Taura Syndrome virus
7	EMS	Early Mortality Syndrome
8	WSSV	White Spot Syndrome Virus
9	HPV	Hepatopancreatic Parvovirus
10	Mm	Millimolar
11	M	Molar
12	°c	Degree Celsius
13	Ha	Hectare
14	Mg	Milligram
15	%	Percentage
16	CFU	Colony-forming unit
17	G	Gram
18	TPC	Total plate count
19	HCL	Hydrochloric acid
20	BOD	Biological oxygen demand
21	H_2SO_4	Sulphuric acid

22	$AgNO_3$	Silver nitrate
23	EBT	Eriochrome Black T
24	DNA	Deoxyribonucleic acid
25	RNA	Ribonucleic acid
26	EDTA	Ethylenediaminetetraacetic acid
27	$MgCl_2$	Magnesium chloride
28	PCR	Polymerase chain reaction
29	TAE	Tris-acetate-EDTA)
30	dNTP	De oxy nucleoside triphosphates (dNTPs)
31	EtBr	Ethidium bromide
32	Ppt	Parts per thousand
33	Ppm	Parts per million
34	TC	Total count
35	TCBS	Thiosulfate–citrate–bile salts–sucrose agar
36	EMB	Eosin methylene blue
37	Mins	Minutes
38	MSA	Mannitol Salt Agar
39	EC	*E coli*
40	OIE	Organization for Animal Health
41	PL	post larvae
42	HLN	Hepatopancreatic and lymphoid organ Necrosis
43	FAO	Food and Agriculture Organization
44	MBV	Monodon baculovirus

45	LSS	loose shell syndrome
46	MSGS	Monodon slow growth syndrome
47	HLN	Hepatopancreatic and lymphoid organ Necrosis
48	EFO	External fouling organisms
49	LSNV	Laem-Singh virus
50	WSD	White Spot Disease
51	SPM	Suspended particulate matter
52	TEM	Transmission electron microscopy
53	PMSF	Phenylmethylsulphonyl fluoride
54	LAMP	Loop-mediated isothermal amplification
55	STE	Sodium chloride Tris-EDTA
56	NaCl	Sodium Chloride
57	µl	Microliter
58	mRT-PCR	Multiplex reverse transcription-polymerase chain reaction
59	RT-PCR	Real-time -PCR
60	ELISA	Enzyme-linked immunosorbent assay
61	AMR	Antimicrobial resistance
62	CIFNET	Central Institute of Fisheries, Nautical and Engineering Training

63	CUSAT	Cochin University of Science and Technology
64	H_2O	Water
65	L	Litre
66	APC	Aerobic plate count
67	SPF	Specific pathogen-free
68	$CaCO_3$	Calcium carbonate
69	V	Voltage
70	mA	Milliampere
71	UV	Ultraviolet
72	bp	Base pair
73	*E. coli*	*Escherichia coli*
74	SS	Salmonella-Shigella
75	DEPC	Diethyl pyro carbonate
76	LAF	Laminar air flow

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ABSTRACT

Shrimp aquaculture is a commercially important industry that experiences prominent losses due to excessive pathogenic invasions, environmental pollution, irregular climatic changes etc. This study focuses on the effect of deviations in the physiochemical parameters on the rate of pathology especially the microbial and viral attack within the shrimp cultured in different farms. To estimate the pathogens in *Penaeus monodon* shrimp samples and the water as well as soil samples from the aquaculture farm were collected from Chellanam and Malipuram areas. Virological tests were conducted for eight viruses which include white spot syndrome virus, yellow head virus, taura syndrome virus, early mortality syndrome, infectious myonecrosis, infectious hypodermal and hematopoietic necrosis virus, hepatopancreatic parvovirus and monodon baculovirus. Bacteriological tests were conducted for the estimation of three different bacterial varieties like *E coli, Staphylococcus aureus* and *Vibrio cholerae*. The results indicated that the physiochemical attributes of water and soil, showed variation from the normal conditions. For instance, there was variation in alkalinity and hardness of the water collected from both the areas under study. Such changes in physiochemical parameters can adversely affect the growth, development and immune responses of shrimps and can be a solid reason for the occurrence of diseases in cultured shrimps. Out of the eight virological tests conducted white spot syndrome virus was tested positive in the sample collected from Malipuram. It is one of the most dangerous and devastating viral diseases found in shrimps. The main reason for occurrence of this virus can be the interconnection between the water sources, as the same disease condition was reported in the nearby farm areas.

The bacteriological tests showed the absence of any coliform bacteria even the *E coli* in the specimen been collected from both areas. Some other bacterial varieties such as *Staphylococcus aureus, Vibrio cholerae* etc were tested to be positive in variable amounts. Total plate count suggested that the specimen from Malipuram is around ten folds contaminated when compared to that from Chellanam. This can also be one of the reasons for the viral detection as the white spot syndrome virus was detected in the specimen collected from Malipuram. The purpose of the study was to identify the pathogens affecting shrimp industry which can help in reducing the economic loss in the industry. Similar kind of studies can help the early diagnosis of diseases in shrimps and can help in the implementation of specific treatment measures as well as precautions for preventing the contamination.

INTRODUCTION

Shrimps have a higher commercial value and are considered a delicacy by many people belonging to different countries. It is the most important traded fishery commodity in many tropical developing countries, like India where it is considered as the most valuable fishery export. Shrimp output is critical for animals, coastal fishing, and biodiversity conservation. Shrimp farming is an aquaculture technique that involves raising shrimp in confined spaces like ponds with sufficient feed, proper ventilation, and a clean atmosphere in order to make them viable (Das et al., 2012). They are cultured in either freshwater or saline conditions but are mainly euryhaline in nature (Das et al., 2012). There are different methods adopted for the culturing of shrimps ranging from traditional farming systems to the most modern techniques (New et al., 1995). India is one of the top five shrimp-growing nations in the world (Jayanthi et al., 2018).

Numerous types of shrimps are cultured throughout the world. It is estimated that there are more than 70 species of shrimps that exist in different parts of the globe. Some of the important species include *Penaeus indicus*(Indian shrimp) found predominantly in Kerala and Karnataka coasts, *Penaeus monodon* (Tiger shrimp), distributed through the east coast and southwest coasts of India, *Metapenaeus dobsoni* (Pink shrimps) seen mainly during July to October months within the west and east coasts of Karnataka and Kerala coasts, *Metapenaeus monoceros*(Brown shrimp) seen abundantly in Andhra Pradesh and Odisha, *Metapenaeus affinis* (King Prawn) seen abundantly from November to May, Penaeus vannamei main aquaculture variety found in China etc (Boyd and Thunjai, 2003).

Penaeus monodon, commonly called Tiger shrimp are the largest prawns in the world and will reach about 33 cm. They have been discovered in Southeast Asia, the Philippines, Australia, and the United States. Because of their bigger size, they are typically more expensive than white shrimp. In the tail region it has black stripes across the dorsal side of the tail in addition to its unusually large size. Its thorax has a spine called the rostrum, one pair of eyes, two pairs of antennae, three pairs of maxillipeds for feeding, and five pairs of walking legs. Except for the telson all other abdominal segment consists of a pair of fins called pleopods on the ventral side. They use the pleopods for forward swimming.

Figure 1*: Penaeus monodon*

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Suborder: Dendrobranchiate

Family: Penaeidae

Genus: Penaeus

Species*: P. monodon*

In India shrimp farming especially *Penaeus monodon* culture is considered a successful business as the continuous supply of feed is assured by the existence of around 300 hatcheries whose capacity is to produce 12 000 million post larvae (PL) annually. However, the sustainability of the sector is still restricted by several problems, among them the most important is relying on the brood stocks being caught in a wild manner whose availability is limited both in seasonal availability and quantity, and thereby they are often infected with pathogens (FAO.2007).

Shrimp farms are largely affected by various pathological invasions. pathogens that infect shrimps can range from different bacteria, viruses, parasites, etc. Pathological invasions in shrimp cultures have adverse social and economic impacts. Viruses have approximately four

times more negative impact on shrimps when compared to all other bacterial diseases. In contrast to viral diseases, the majority of diseases caused by bacteria and other parasites can be prevented to a certain extent by efficient management of shrimp culture farms (Flagel et al.,2012).

Shrimp viruses frequently cause no noticeable signs of illness in shrimp, particularly in their natural environment. Some of these viruses can become more virulent and cause substantial economic loss through mortality or stunted development in stressful settings like culture systems. However, some of them, like WSSV, is extremely fatal.

One of the major challenges faced by shrimp aquaculture is the excessive loss due to viral diseases like white spot syndrome, yellow head disease, and Taura syndrome. There are numerous instances of shrimp diseases spreading globally as a result of the export of live shrimp for aquaculture. With respect to certain studies, there are more than 20 viruses being reported to infect marine shrimp. Many of these were not been associated with clinical signs of disease and some of them were viewed only by electron microscopy as they are poorly characterized. Seven viral pathogens of marine shrimp are currently listed by the World Organization for Animal Health (OIE) as those which causes aquatic organism diseases and two are under study for listing (OIE 2008); (Lightner et al., 2011).

There are both infectious and non-infectious diseases that effect shrimp development and growth. Some of the infectious diseases are caused by virus such as monodon baculovirus (MBV), hepatopancreatic parvo-like virus (HPV), infectious hepatopancreatic and lymphoid organ necrosis (HLN) etc and some bacterial diseases are systemic vibriosis, a luminescent bacterial disease. On the other hand, there are certain fungal disorders such as larval mycosis and parasitic disorders like protozoan fouling, microsporidiosis etc. Non-infectious disease which has prominent effects on shrimp culture is termed as a soft-shell syndrome which infects mainly Penaeus indicus (Lightner et al., 1993).

Other than microbial and virological diseases there are several other pathogens that have adverse effects on the shrimp culture. Some of the fungal, protozoan, and parasitic manifestations found in shrimps are larval mycosis, black gill disease, microsporidiosis, etc. Larval mycosis is filamentous of genus Lagedenium species whereas black gill disease is caused due to fusarium species and microsporidiosis is an infection due to nosema species, thelohania, and pleistophora species (Uddin, et al., 2013).

According to several studies, approximately 60% of disease losses in shrimp aquaculture have been caused by viral pathogens and 20% by bacterial pathogens. When compared to these pathogens the impact of fungi and other parasites on shrimps are very much lesser (Baticados et al., 1990). Vibrio species are the most important bacterial pathogens, while the importance of viral pathogens has changed since 2003 when the formerly dominant giant tiger or black tiger shrimp *P. monodon* was replaced as the dominant cultivated species by genetically selected stocks of the American white leg shrimp Penaeus (*Litopenaeus vannamei*) (Flegel et al.,2012).

White spot syndrome virus (WSSV) and yellow head virus (YHV) are the deadliest in both species. According to some accounts, the microbes implicated in shrimp illness can be either opportunistic or pathogenic. Opportunistic bacteria have the ability to cause infections under unfavourable environmental circumstances. Bacterial diseases in shrimp can result in decreased growth, death, cuticle lesions, necrosis, muscle opacity, gill colour change, loosened cuticle, pale gut, unsteady behaviour, and retracted feed absorption (Johnson and Sterling ,1995).

In shrimp and prawn farms, changes in temperature, pH, salinity, and alkalinity can be regarded as the primary causes of post-larval disease epidemics. Conduction of quality parameters is important enough to check whether the environmental conditions are suitable for the growth of tiger shrimps since any kind of deviation from the normal rates can adversely affect the growth by increasing the environmental stress. The guidelines developed by the World Animal Health Organisation for the movement of live animals for aquaculture, frozen crustaceans for human consumption, and the regulations implemented by some shrimp importing regions to focus mainly on assessing the risk factors and also to determine the effect of viral diseases on the international trade (Karunasagar et al., 2012).

Shrimp pathology, considered as a supportive profession to penaeid shrimp aquaculture, is assumed to have started more than 30 years ago. In most of the primitive publications, simple diagnostic methods such as examination of wet mounts of diseased tissue by phase or bright-field microscopy were used to describe the first known diseases and pathogens affecting cultured shrimps and wild varieties of shrimps. Fungal and bacterial isolates from shrimp were characterized by using classic microbiological techniques (Lightner and Redman.,1998).

A major catastrophe can be avoided with early discovery and swift action to contain and cure the illness. Facilities for shrimp that are discovered to be harbouring an exotic pathogen need

to be promptly separated and treated before the arrival of fresh shrimp. The most effective method to avoid diseases or decrease disease-related losses is to improve farm administration and strengthen the shrimp's immune system. The shrimp aquaculture industry's major concern is shrimp diseases (Kannappan, 2022).

The disease is a major problem in the prawn farming sector. It is challenging to avoid and manage shrimp illnesses because the link between inherent immune mechanisms and environmental adaptation mechanisms is poorly understood. Recently, there has been a lot of focus on the innate immune system of shrimp, and preliminary descriptions of the roles played by the humoral and cellular immune responses have been made. In an effort to better understand the interplay between the innate immune response, the environmental stress response, and illness, the impact of environmental stress in shrimp disease has also lately been studied (Chen et al., 2019).

A complicated connection exists between shrimp illnesses and both the innate immune response and the environmental stress response. Immunization treatment cannot be used to avoid and manage shrimp illness because crustaceans lack an adaptive immune system. However, ecological methods can be used to manage shrimp illnesses. Shrimp illnesses are greatly diminished by using these methods, which are founded on the natural immune reaction and the environmental stress response (Chen et al., 2019).

The rate of shrimp pathology is found to increase exponentially due to several reasons, out of which environmental pollution is considered one of the major causes. Most often shrimp farming is done in brackish waters, ponds, etc. They get easily polluted by the chemicals and fertilizers that seep from the soil as well as any other sources. Shrimps can be affected not only by bacteria and viruses but can also be adversely affected by the invasion of several sought of insects and other harmful organisms. There are several studies have been carried out to determine the impact of different virus and bacteria on shrimps, however, studies which combine both the evaluation of physiochemical parameters, as well as the detection of bacterial and virological organisms in the *P. monodon,* is very rare. So, the current study is carried out to determine the viral and bacterial content within the *P. monodon* variety in response to the deviations in the physiochemical attributes of soil and water.

AIM

The current project mainly focuses on the early detection and diagnosis of bacteria and viruses within shrimps that are being cultured in the farms of Malipuram and Chellanam areas with respect to the variations of the physiochemical attributes of their environment.

OBJECTIVE

The three major objectives of present study were;

I. To study the microbial growth in Farmed shrimps
II. To determine the presence of the virus in *Penaeus monodon*
III. To conduct a comparative study of the physiochemical characteristics of water and soil and the presence of pathogens in two different shrimp farm areas such as Chellanam and Malipuram.

RELEVANCE

The economics of shrimp culture influence the socioeconomic status of coastal villages and is a lucrative fishery product in many tropical developing nations. In terms of worth, shrimp is one of the most significant export fishery products. The current study is an investigation on the presence of pathogens on tiger shrimps and its relation with the physiochemical characteristics of water and soil. Studies on viral and bacteriological infections found in shrimps are highly relevant since consumption of infected shrimps can lead to severe health problems in human beings. Apart from the health issues, the presence of any viral infections such as white spot syndrome virus can lead to massive death of shrimps and can cause an ultimate economical loss for the farmers who considers shrimp farming as their livelihood. This research work aims to identify probable diseases in shrimps so that early treatment measures and precautions to prevent the spread of the disease can be taken by the farmers in the region.

REVIEW OF LITERATURE

VIROLOGICAL TESTS

Over the last two decades, more than 15 distinct viruses have been discovered in Penaeid shrimp (Fulks et al., 1992; Bower et al., 1994). Many viruses have been identified to affect larvae and adolescents and can be quite particular in terms of which species are infected. Insufficient space, weather changes, low oxygen levels, and excessive amounts of pollutants can all cause virus replication. It is obvious that novel viruses, such as the gut virus, will be discovered in the coming years. Because shrimp aquaculture is being brought to Africa and Madagascar, where novel illnesses may be met, it is entirely conceivable that some of the worst viral outbreaks are yet to come. As per the study conducted by Lundin (1996), understanding is inadequate to make any assumptions about the scope of these diseases. The only temporary solution to viral illness is to enhance health management to reduce the likelihood of infection (Lundin and Carl Gustaf,1996).

Five distinct viruses were examined for their influence and impact on the commercial farming of the black tiger shrimp (*P. monodon*) in Thailand in the research by Flagel et al. (1997). Some of these viruses have the potential to infect other types of crab species as well as penaeid shrimp species. Some of these viruses have been discovered in farmed shrimp from Australia and the Western hemisphere as well as various Asian nations. The five viruses were the infectious hypodermal and hematopoietic necrosis virus, yellow-head virus, hepatopancreatic parvo-like virus, and monodon baculovirus, listed in decreasing order of their economic effect on the Thai seafood industry. The goal of this study was to collect recent work on these viruses and to suggest future research paths that could be very helpful in the endeavour to create a viable shrimp business (Flagel et al.,1997).

In the previous study by Pramod Kiran et al., (2012), they discuss the different types of diseases found in shrimps and the methods adopted for health management related to it. In their view, health management has always played an important role in modern shrimp culture. Shrimps have a primitive immune system compared to fishes and are reared in environments where several pathogens are naturally present. According to World Organization of Animal Health

(OIE) has listed 5 viral diseases important for shrimp they are white spot syndrome virus (WSSV), infectious myonecrosis virus (IMNV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), taura syndrome virus (TSV), yellow-head virus (YHV). Among them, the most dangerous and hazardous disease is considered to be the white spot disease syndrome virus (Pramod Kiran et al., 2012).

The majority of the material in the study conducted by Tandel et al., (2017) is about new emerging pathogens that endanger India's shrimp farming business. Other significant pathogens that infect *P. monodon and L. vannamei* include infectious hypodermal and hematopoietic necrosis virus (IHHNV), monodon baculovirus (MBV), and hepatopancreatic parvovirus (HPV). In late 1998, an emerging illness known as loose shell syndrome (LSS) was described in India. Monodon slow growth syndrome (MSGS), a component of which appears to be the laem-Singh virus, is a more recent illness of *L. vannamei* in India (LSNV) (Tandel et al., 2017).

Based on the studies conducted by Vaseeharan et al., (2003), at nine distinct *Penaeus monodon* rearing hatcheries in India, the monodon baculovirus (MBV), external fouling organisms (EFO), and microbes (especially vibrio species) were observed in 1996–1997. In comparison to uninfected post larvae, MBV-infected post larvae and their surroundings had greater levels of vibrio-like bacteria. Both MBV-infected and MBV-uninfected post-larvae were found to have an overwhelming preponderance of presumed *Vibrio harveyi* and *Vibrio anguillarum* in the tank water used for raising them.

The research work performed by Chakrabarty et al., (2014) and colleagues estimated the occurrence of WSSV in *P. monodon* in three different seasons both qualitatively and quantitatively during the years 2011 to 2013 along the west coast of India (Chakrabarty et al.,2014). White spot syndrome virus (WSSV), is said to have appeared for the first time in the 1990s in Taiwan, where it spread rapidly to shrimp-farming areas all over the world, later it became one of the major pathogens found in cultured shrimp (Lo et al., 1996). The most dangerous virus for black tiger shrimp and animals is the white spot syndrome virus (WSSV) (Phillips et al., 2016). It's host range is very broad, infecting not only the cultured shrimp but also other invertebrate aquatic organisms such as crayfish (Prayitno et al., 2022).

The virus has the ability to cause 100% accumulative mortality within 2–10 days in farmed shrimps. According to the studies conducted by Wang et al., (2005), antisera against six white spot syndrome virus (WSSV) envelope proteins was used in neutralization assays conducted in vivo. The results showed that the virus infection could be significantly neutralized or delayed

by using antibodies against three WSSV envelope proteins such as VP68, VP281, and VP466 (Wang et al.,2005).

Through bivariate analysis, Talukder et al., (2021) were able to identify nine significant risk variables for white spot syndrome and showed the 2018 WSSV frequency and causes of WSSV outbreaks in Bangladesh (Talukder et al.,2021). White Spot Disease (WSD), induced by the white spot syndrome virus (WSSV), causes extensive mortality and commercial losses in almost the entire Asian shrimp farming business. The spread of illness incidence, on the other hand, is uneven and is likely to be determined by several kinds of management, environmental, and socio-ecological parameters. In this research, 233 farms in southwest Bangladesh, the primary shrimp farming zone, were examined to generate a list of pond kinds, culture methods, and farming practices. To identify important risk factors, variables possibly adding to WSD incidence in the present shrimp harvest were first filtered using univariate analysis and then analyzed using multivariate logistic regression (Hasan et al., 2020).

PHYSIOCHEMICAL PARAMETERS INFLUENCE ON SHRIMP CULTURE AND PATHOLOGY

Numerous studies have been conducted on the environmental effects of shrimp farming in general since ancient times, with only a small amount of work done in Sri Lanka. The study done by Senarath et al., (2001) study offers some essential context for brackish water shrimp aquaculture in Sri Lanka. It concentrates quickly on the sustainability and development of shrimp aquaculture, as well as the industry's present state. The emphasis is on two general issues: the effects on the climate and the current wetland habitat. These effects are thought to be the primary reasons for the industry's slow development and broad public outrage. The practice of modern environmental and ecosystem administration is addressed. Finally, management and long-term growth strategies are discussed (Senarath et al., 2001).

The reasons for pathogen growth and dissemination in shrimp farming are reviewed and discussed from an ecological standpoint in the article by Kautsky et al., (2000). Due to a lack of pure water supply and inadequate waste clearance metabolites will become overloaded causing the degradation of ecosystem, and shrimp become agitated by poor water quality, making them more susceptible to disease. Excessive variations in abiotic variables such as

oxygen, salinity, and temperature may also raise stress and disease vulnerability (Kautsky et al., 2000).

The bacterial communities in shrimp ponds located in Indonesia with various population levels were identified in the research by Alfiansah et al. (2018). In moderate density/semi-intensive (40 post-larvae m-3) and high density/intensive shrimp ponds (90 post-larvae m-3), the water quality, including physical variables, inorganic nutrient levels, and cultivated heterotrophic bacterial abundances, including potential pathogenic vibrio, were evaluated throughout the shrimp cultivation period. The variations between semi-intensive and intensive environments regarding suspended particulate matter (SPM), salinity, chlorophyll a, pH, and liquid oxygen were substantial (Alfiansah et al., 2018).

The following article by renders Moullac et al., (2000), is an attempt to analyze and evaluate the available information discussing how external factors affect the immune response in Crustacea. With regard to the shrimp that have been raised, importance has been placed on natural ecosystem variations, chemical contaminants, and physicochemical changes (Moullac et al., 2000). Given its immediate impact on metabolism, consumption of oxygen, growth, maturation, and their existence, water temperature is probably the most prominent environmental factor (Hennig and Andreatta, 1998). Other natural variables like salt and water oxygenation are influenced directly by temperature.

In order to identify alternatives for sustainable production, the study by Sousa et al., (2013) concentrates on the environmental and socioeconomic consequences of semi-intensive and intensive shrimp aquaculture in the littoral area in North Eastern Brazil. The results of this research demonstrate that for the Brazilian seafood business to expand ecologically, improved management and development policies are needed (Sousa et al., 2013).

The findings from the research work proposed by Zhang et al., (2022) demonstrated that various microalgae significantly affected both shrimp efficiency in growth and the natural characteristics of the rearing water. To provide an optimal ecological environment for shrimp culture in this context, experts are currently investigating ways to enhance water quality based on the regulation of bacterial or microalgal populations in aquaculture water (Alonso-Rodriguez et al., 2003). This will assist in avoiding disease outbreaks and increase the success rate of aquaculture (Janeo et al., 2009; Neori, 2010).

Microalgal ecological regulation technology is developing biotechnology that organically incorporates algal biology, algal ecology, and aquatic ecosystems (Beardall and Giordano,

2002). It is the most efficient way to improve water quality, maintain an optimized water environment, and implement ecologically healthy shrimp breeding programs (Cao et al., 2007; McCausland et al., 1999).

The environmental impacts of shrimp aquaculture in Bangladesh's southwest coastal region were investigated in this study by Chowdhury et al., (2011), along with the state of the water, sediment, and soil. It was found that the water quality used in aquaculture techniques was suitable for shrimp's best growth and longevity (*Penaeus spp.).* The findings of this study demonstrated that the long-term salinization of soil is a consequence of using saline water for shrimp farming. It poses a significant threat to Bangladesh's ability to sustain coastal shrimp cultivation and coastal development as a result (Chowdhury et al., 2011).

DIAGNOSIS OF PATHOLOGY IN SHRIMP

A study conducted by Mari et al., (1993), MBV (monodon baculovirus), pathogenic for the shrimp *Penaeus monodon*, was recognized as a probable baculovirus based on the ultrastructural characteristics in TEM. The complete study was done to get information related to the morphological characteristics of monodon baculovirus which is a prominent virus that infects the tiger shrimps. The identification and study of these types of viruses are really relevant in early diagnosis and prevention of certain diseases in shrimps (Mari et al.,1993).

According to the research work presented by Lundin and Carl Gustaf (1996), the short-term strategy for dealing with bacteria was used which involves the use of antibiotics as well as improved pond cleaning and an increase in water exchange within the shrimp farms. Lundin and Carl Gustaf (1996), did a worldwide assessment of the shrimp illness They reported several viral, bacterial, rickettsia, fungal, and protozoan diseases. The primary emphasis of this article has been on how to handle the problem of rampant shrimp illness (Lundin and Carl Gustaf,1996).

A new method for extraction of viral DNA and isolation of prawn baculovirus was developed through the studies conducted by Wang et al., (1997). No density gradient centrifugation, ultracentrifugation or phenol-chloroform extraction steps were involved in this study. Degradation of DNA and RNA was done using DNase and RNAase whereas, on the other hand, Protein degradation was prevented by using Phenylmethylsulphonyl fluoride (PMSF). Intact viral DNA was obtained by lysing nucleocapsids. Restriction fragments were shown in

a clear manner within the agarose gel, as the viral DNA was digested by using restriction endonuclease and separated with electrophoresis (Wang et al., 1997).

Based on the studies conducted by Savan et al., (2004) a novel, rapid and very sensitive method was utilised for the diagnosis of wide range of disease in shrimp aquaculture, named as Loop-mediated isothermal amplification (LAMP). The presence of WSSV in the heart, stomach and lymphoid organ from infected shrimp was detected by using standardized LAMP procedure. The study has developed a diagnostic procedure which is a rapid and highly sensitive for WSSV detection in shrimp which increased the rate of accuracy in detection of early diseases (Savan et al., 2004).

According to the studies conducted by Wang et al., (2005), antisera against six WSSV envelope proteins were used in neutralization assays conducted in vivo. The results showed that the virus infection could be significantly neutralized or delayed by using antibodies against three WSSV envelope proteins such as VP68, VP281 and VP466 (Wang et al.,2005).

A short review on infectious viruses in shrimp culture method and the commonly used diagnostic methods, conducted by Ganjoor (2015) stated that methods which are applied for the detection viral disease in shrimp is different and some of them include, Histology (staining-Light Microscopy), TEM (Transmission electron microscope), Non-nested PCR, Nested PCR, Multiplex PCR, Multiplex reverse transcription-polymerase chain reaction (mRT-PCR), Real-time RT-PCR, Multiplex RT-nested PCR, Mini array, Single-step multiplex PCR, Single PCR, ELISA (Monoclonal antibody assay based test), PCR–ELISA, Fluorescence microscopy, In situ hybridization (A type of Nucleic acid based test) and Monoclonal antibody assay based tests etc(Ganjoor,2015).

Through the research work done by Lavilla-Pitogo et al., (1995), the creation and application of quicker detection techniques like the indirect fluorescent antibody technique, monoclonal antibodies, and other enzyme immunoassays are required for better monitoring and surveillance. Diagnosis has traditionally been primarily accomplished through conventional bacteriology and histopathology. Due to the conflict between the use of chemotherapy and the associated environmental risks, many studies are now focusing on non-medicinal approaches to address the issues associated with infectious illnesses. (Lavilla-Pitogo et al., 1995)

In compliance to the study performed by Seethalakshmi et al., (2021), nanoparticles, biofilm-based immunizations algal extracts, phytobiotics, probiotics, prebiotics, and synbiotics are just

a few of the innovative and successful treatments that the fields of nanotechnology and biotechnology have suggested in recent years to fight contagious illnesses. As they are biologically derived, algal products, phytobiotics, probiotics, prebiotics, and synbiotics are acceptable to use in aquaculture environments.

BACTERIOLOGICAL TESTS

Concerning certain research works, Shrimp are susceptible to a wide range of bacterial infections, commonly as an opportunistic result of a viral illness or environmental duress. The following groups have seen the majority of disease epidemics to date: bacteria such as *Leucothrix sp*., *Pseudomonas sp*., Citinoclastic bacteria, Luminous bacteria, and Vibrio bacteria. In unhygienic hatcheries and other high-stress circumstances, bacteria have been particularly disruptive. Numerous of these bacteria are present naturally throughout the tropics and can affect a variety of crustacean species (Fulks et al, 1992; Bower et al, 1994).

In compliance with the research work done by Lavilla-Pitogo, bacterial diseases have started to restrict the development of penicillin culture systems over the past five years, with the intensity and impact of their effects directly correlated with the industry's expansion. *Vibrio alginolyticus, V. cholerae (non-01), V. damsela, V. fluvilis, V. nereis, V. splendidus, V. tubiashii, V. vulnificus, V. parahaemolyticus, and V. harveyi* are just a few of the Vibrio species that have been linked to penaeids (Lavilla-Pitogo et al., 1995).

A quantitative study carried out by Vaseeharan et al., (2003) of all the Vibrio-like bacteria in hatcheries showed that post-larval mortality happens when the Vibrio-like bacteria reach 2*102 CFU. From shrimp eggs, post larvae, raising tank water, source seawater and feed, total cultivable heterotrophic bacteria, Vibrio-like bacteria, and presumed *Vibrio harveyi, Vibrio anguillarum,* and *Vibrio vulnificus* numbers were obtained (Artemia nauplii and microencapsulated feed). The presence of Vibrio-like bacteria in Artemia nauplii made it obvious where these pathogenic bacteria might have originated and that will be in the nursery settings (Vaseeharan et al., 2003)

Kusumaningrum et al., (2015) studied the bacterial and fungal diseases of shrimp post larvae. *Vibrio, Pseudomonas, Aeromonas, Alcaligenes, Bacillus, Staphylococcus, Hafnia, and Fusarium* are likely the genera of microorganisms that were isolated.

Prabina et al., (2022) identified and characterized vibrio from infected shrimps (*Litopenaeus vannamei*) of two cultured farms, to compare their pattern of antibiotic resistance, and to determine whether AMR is of plasmid borne or chromosome-mediated. Bacterium detection in the study by Shaekh et al., (2013) and fellow researchers, endeavour, morphological, physiological, biochemical, and genetic characterizations were carried out. Gram-positive staphylococci This study was mainly focused on searching for antagonistic bacteria by morphological, physiological, and biochemical tests, which may be used to control emerging pathogens (Shaekh et al., 2013).

METHODOLOGY

STUDY AREA

The current study on "Bacteriological and Virological Assessment of Cultured *Penaeus monodon* in Vypin and Chellanam areas "was conducted in two different aquaculture farms in Chellanam and Vypin. In a precise manner, the area chosen for the conduction of study are Malipuram regional area of Vypin and Chalipuram, the beginning area of Chellanam.

Chellanam is a hamlet in the Indian state of Kerala and near the city of Kochi. Most of the people make their living from fishing and agriculture. The sample for the current study was collected from a brackish water farm with shrimp culture where different types of shrimps were cultured. The farm was connected to the sea by a river and thereby the salinity of water was relatively lesser than marine condition (Fig 1).

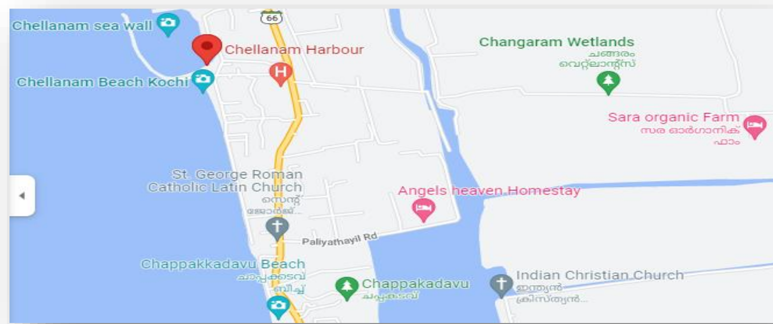


Figure 2: Map of Chellanam shrimp culture farm

Malipuram is a small village found within the Vypin area. It is also considered as a tourist spot where there are numerous aquaculture farms visited by many tourists. The village is well known for the availability of shrimps and varieties of fishes been cultured in this region. For the current study, many a number of Penaeus monodon were collected from one of the farms which was interconnected with other similar aquaculture farms. (Fig 2).

Figure 3: Map of Malipuram shrimp culture farm

For the purpose of the current study, it was necessary to measure the quality parameters of both soil and water along with the bacteriological and virological tests. All sought of quality tests such as estimation of salinity, alkalinity, dissolved oxygen, PH, temperature and hardness as well as total plate count of bacteria was done in CIFNET (Central Institute of Fisheries, Nautical and Engineering Training). The virological tests were carried out in CUSAT (Cochin University of Science and Technology). Eight different viruses were tested in CUSAT. As part of the research work, it was also necessary to conduct a bacteriological analysis. Three different bacteria were selected to be analysed in the body of shrimp. The specimen was given for bacteriological analysis at CMFRI (The Central Marine Fisheries Research Institute).

SPECIMEN COLLECTION

Study was conducted on *P. monodon* commonly known as Tiger shrimps. The specimen was required for three different purposes as part of the research. Shrimps required for each test were collected from respective aquaculture farms by the cooperation of farmers in that region. The farmers used traditional fishing nets to capture shrimps. Shrimp farms chosen for study were located in Malipuram and Chellanam. Both Total plate count conducted in CIFNET and virology tests conducted in CUSAT required frozen samples whereas for conduction of bacteriological analysis in CMFRI it required live samples. All samples after collection were neatly packed in a zip lock bag in order to prevent any sought of contaminations. For transportation, an icebox was used so that the temperature can be regulated.

PROCEDURE

WATER QUALITY PARAMETERS

SALINITY

The experiment was started by filling the burette with 0.01N silver nitrate solution. Then 10ml of water sample A was taken in a conical flask and a few drops of 5 percentage potassium chromate solution was added to it. The water samples were titrated against silver nitrate solution. The end point will be the appearance of a brick red colour. Titration was continued until the concordant values were obtained. A minimum of two times titration should be done. The same procedure is repeated with the water sample taken from both the areas.

Salinity was determined using the following equation;

$$1ml \text{ of } AgNO_3 = 2 \text{ mg of chloride}$$

$$\text{'a'ml of } AgNo_3 = 2 \text{ 'a'mg of chloride}$$

$$\text{Volume of } AgNo_3 = \text{Volume of } AgNo_3 \text{ in titration- volume of AgNo3 in blank}$$

$$\text{Chlorosity} = 2\text{'a'}$$

$$\text{Chlorinity} = \text{chlorosity /density of } H_2O$$

$$\text{Salinity} = 0.03 + (1.805 * \text{chlorinity})$$

ALKALINITY

For this experiment 100ml of sample was taken in a 250ml conical flask and 2 to 3 drops of phenolphthalein indicator is added. If no colour is produced, the phenolphthalein alkalinity can be considered zero. As pink colour was developed it was titrated with 0.1 N hydrochloric acid till it disappears. The volume of HCl being used was noted. Then 2 drops of methyl orange were added to the same flask, titration was continued till the orange colour of solution changes to pink. The volume of HCL used is noted. Both phenolphthalein alkalinity and total alkalinity are calculated. The same procedure was followed for the water sample collected from both Chellanam and Malipuram. Alkalinity was determined using the following equation;

$$\text{Alkalinity of sample in mg } CaCO_3/L = (\text{Volume of HCL consumed /Volume of sample taken}) * 50 * 1000$$

DISSOLVED OXYGEN

The sample was collected in a BOD Bottle taking care to avoid any bubble formation. Sample was filled to the neck of the bottle. Be 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. 1 ml of manganous sulphate followed by 1 ml of alkali iodide azide solution was added to it. While adding these chemicals, keep the tips of the pipette below the liquid level. The stopper was placed carefully to exclude air bubbles and mix by inverting the bottle repeatedly for at least 15 minutes. Stopper was removed carefully and immediately 1 ml of Conc. H2SO4 was added into it, the bottle was closed and with gentle inversion it was mixed until the precipitate completely dissolve. Using starch as a signal, 50 ml of the bottle's contents were titrated with sodium thiosulphate solution. The shade of blue fades to colourless at the conclusion. The

starch indicator is usually added towards the end of the titration when a straw pale colour is obtained. This entire process was done for the estimation of dissolved oxygen of both water samples. Dissolved oxygen was determined using the following equation;

Oxygen content of sample = (8× volume of sodium thiosulphate used × normality of sodium thiosulphate × 1000) /volume of sample used

HARDNESS OF WATER

In order to determine the hardness of both samples, 50 ml of sample was taken and mixed well in a conical flask. 1-2 ml buffer solution was added into it. After addition of a pinch of EBT it was titrated against standard EDTA (0.051 M) till wine red colour changes to blue. Hardness was determined using the following equation;

Molarity of titrant (EDTA) = 0.051M

Total hardness = (Volume of titrant* molarity of titrant × 1000×100)/ volume of sample taken

TEMPERATURE

There are different methods for determining the temperature of a water sample. In the current work a laboratory thermometer was used for the purpose of measuring the temperature. After taking a laboratory thermometer the initial values of mercury readings are noted. First the water sample from Chellanam was taken in a beaker. Then the thermometer was placed in a vertical manner without touching the bottom of the beaker but almost in the middle of water. It was kept in stationary manner for almost 10 minutes. After 10 minutes the temperature in the thermometer was noted. This whole process was repeated in water sample collected from Malipuram also.

pH

A pH probe is used to detect the pH of water samples. Both the sample solutions were taken in different beakers and then PH probe was dipped into it. It was kept in a stationary manner for 5 minutes. The readings indicating the PH of solution can be seen on the instrument.

SOIL QUALITY PARAMETERS

REDOX POTENTIAL (EH)

In order to determine the Eh of the soil a Eh probe was used. It appeared to be in the form of a rode shaped tube-like structure. For the process of estimation, probe was kept inside the soil taken in a beaker. After a period of 5 minutes, the probe will show a value on it and it denotes the Eh value of soil. The same method was followed for both samples of soil.

TEMPERATURE

Temperature of soil was estimated in a similar as that of the temperature of water samples. Here also a laboratory thermometer was used. It was colloquially known as lab thermometer also. After taking the samples of soil in two different beakers the laboratory thermometer was immersed slowly into the beaker in such a manner that it doesn't touch the bottom surface of the beaker but is kept in an upright position is hold firmly using the hand to stay in a stationary form for almost 10 minutes. The temperature on the thermometer is noted.

PH

The next parameter to be measured in soil was pH of the soil. pH probe was used for this purpose. In this method, two beakers containing the soil samples from each of the region were collected and then the Ph probe was slowly immersed into the soil. It was allowed to stand for a particular period of 5 minutes of time. The value of pH will appear on the probe.

VIROLOGICAL TESTS

In the current work conduction of virological tests in *Penaeus monodon* is really important to identify whether any virus is present in it. Eight different types of viral tests were conducted. Tests for White spot syndrome virus, yellow head virus, taura syndrome virus, early mortality syndrome, infectious myonecrosis, infectious hypodermal and hematopoietic necrosis virus, hepatopancreatic parvovirus, Monodon baculovirus were conducted. All virological tests were done in CUSAT. The virus detection was done using the following procedures

1. ISOLATION OF DNA

DNA isolation was done by DNA express kit digestion buffer. In the initial step, fresh MCT tubes 2 in number were taken and marked as sample1 and 2. The specimen collected from both regions were taken and dissected in their gill region carefully using a dissection blade. A very minute number of tissues were taken and inserted into each of the MCT tube.1ml of STE buffer was added into it.

STE BUFFER COMPOSITION

5M NaCl – 20µl

1M Tris HCL-200µL

0.5M EDTA- 1100µl

Milli Q – 680µl

Then using a homogenizer, the tissue was homogenized very well. STE buffer is considered as a digestion buffer. A new Eppendorf tube is taken and sample ,STE buffer 920µl ,50µl SDE and 30µl of proteinase K were added into it. It was mixed well and was kept in water bath for about 1 hour. To that mix equal amount of DNA express was added. Then it is centrifuged at 10-12000rpm by keeping in a centrifuge. This is accomplished by rapidly rotating the fluid within a receptacle, separating fluids. By using a tip, 500µl supernatant in a new 1.5ml MCT was taken. Then 100 % chilled ethanol of about 1ml is added to it. Then it is kept at -20 degree Celsius for 10 minutes. It is spun for 10 minutes at 10000 speeds. Then ethanol was decanted and add 500µl of 75% ethanol was added to it. Then it is centrifuged and decant again. The precipitate was dried and 20ul Milli Q was added to it. Then dissolved it and stored at -20 degree Celsius. Thus, DNA was extracted.

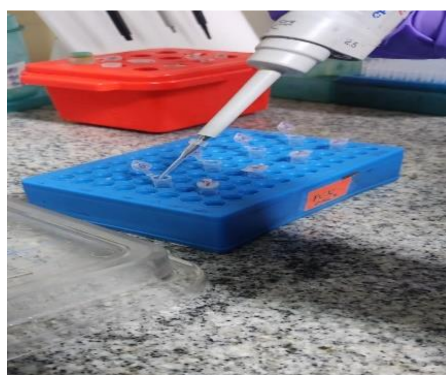


Figure 4 : Isolation of DNA

1. ISOLATION OF RNA

In the current experiment, from shrimp gills, around 50-100mg tissue per ml of TRI reagent is taken. Then DEPC treated needles dipped in 10% cold sodium citrate made in DEPC treated water is placed. Homogenizer along with TRI reagent is placed in ice. Tissue is then drawn with the anticoagulant dipped needles into the homogenizer. It is homogenized thoroughly and more TRI reagent proportionate to the amount of sample to retain the pink colour is added. Then it was transferred into fresh 2ml MCTs. 0.2 ml of chloroform (free of isoamyl alcohol/addictive’s)/ml of TRI reagent. The samples were covered tightly and shacked vigorously for 15sec. It was allowed to stand for 15 minutes at room temperature (maximum of 30 minutes). The resulting mixture was centrifuged at 12000rpm for 15 minutes at 4 degrees Celsius. Three phases were developed. Red organic phase indicates the presence of protein, interphase consist of DNA whereas colourless upper aqueous phase indicates the RNA. This colourless aqueous phase was transferred to a fresh 1.5ml MCTs Both interphase and organic phase were stored at 4 degree Celsius for DNA and protein isolation. 0.5ml of TRI reagent was added into it. Then the MCT tubes were turned for 5 to 6 times in a spinner. Allow the sample to stand for 10 minutes at room temperature. The mixture was then spun at 12000rpm for 10 minutes at 4 degrees Celsius. RNA pellets develop on the tube's edges and bottom. The supernatant formed is decant immediately after centrifugation. The RNA pellets formed are washed by adding 1ml of 75% ethanol/ml of TRI reagent (75% ethanol – 79ml of 95% distilled ethanol and 21ml of DEPC treated water). The samples are then vortexed and pool into a single tube. It is rotated for 10 minutes at 4 degrees Celsius at 12000rpm. The supernatant formed 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 maximum 30minutes).20µl of DEPC treated water is added to the MCTs and vortex thoroughly. It is spined in a mini centrifuge. It is then kept in ice. For the Rna isolation a cDNA synthesized with a specific composition.

cDNA synthesis (one-strand synthesis):

RNA inhibitor =0.5µL

dNTPs(10mM) =2µL

Oligo dt = 4µL

RT buffer =2µL

MgCl2(25 mM) =1.6µL

RT enzyme =0.5µl

Program - Oligo dT(42°C; 1hr

Run in 1% agarose gel

Figure 5: Isolation of RNA with the help of LAF

QUANTIFICATION OF RNA

First the instrument was switched on. Now on the screen of the system select nucleic acids. No specification for DNA and RNA is required since both are together known as nucleic acids. Then at an option of 'blank' is clicked and then the value of blank is noted. Now an opening could be found within the instrument. It is carefully opened and a red coloured spot light can be found on it. Using a wipe specifically used for wiping this device, the lighted spot is wiped and then a gun pipette with microtip is used to take the RNA from the first sample to be introduced correctly into this spot. After the introduction of the first sample the opening was closed and then 'sample 'option was clicked. Now the concentration of first sample is obtained. In the same method the quantification of RNA of both the sample were done.

Figure 6: Quantification of RNA

3. POLYMERASE CHAIN REACTION (PCR)

For the conduction of PCR each of the virus requires a particular condition to be set on the system only then it can enable in the proper amplification of the gene can be possible. There are eight different virological tests that are conducted and each of them had separate conditions. To begin with each Eppendorf tube was taken and 5ul of emerald green, 3.8µl distilled water,0.4µl forward primer and reverse primer of each particular viral detection were added After addition of primers the respective genes (DNA and RNA) of 0.4µl quantity is added to it. After the addition of each example, all Eppendorf tubes were subjected to spinner, to spin the contents and then it was taken to the PCR instrument. After switching the PCR instrument on , the option 'files' was selected. Then press load option, select any of the programme from the ones that appear on the screen . Make necessary corrections in the conditions over there depending on the viral gene that is to be amplified. Press exit. A question arises whether to save press 'yes' by enter. Then all the Eppendorf tubes being labelled well were introduced into the PCR. Then press enter for 0.2 quantity in a question that is found on the screen. Type 10µl exactly then press enter and click start button. Then 'opt' is clicked to check the time required for the completion of reaction. This same process was repeated for all the different virus with both the samples.

TABLE 1: Parameters for PCR testing for each virus

	White spot syndrome virus	Infectious hypodermal and hematopoietic necrosis virus	Taura syndrome virus	Early mortality syndrome	Infectious myonecrosis	Yellow head virus	Hepato pancreatic parvovirus
Initial denaturation	95°C 5 minutes	95°C 3 minutes	94°c- 2 minutes	95°c 5 minutes	95°c 3min/2min	94°c 2 minutes	95°c 5minutes
Denaturation	95°C 30 seconds	95°C 30 seconds	94°c- 45 seconds	95°c 30seconds	94°c 45 seconds	94°c 30 seconds	95°c- 30 seconds
Annealing	55°C 30 seconds	55°c 30 seconds	60°c- 45 seconds	60°c 30seconds	68°c 45 seconds	58°c 30 seconds	55°C 30 seconds

Extension	72°C 60 seconds	72°C 60 seconds	60°c- 60 seconds	72°c 60seconds	72°c 60 seconds	72°c 30 seconds	72°C 60 seconds
Repeat	Go to step 2, repeat 34cycles	step 2, repeat 34 cycles	Go to step2 repeat 39 cycles	Step 2 repeat 34 cycles	Go to step2 repeat 39 cycles	Go to step2 Repeat 39 cycles	Go to step 2 repeat 34cycles
Extension final	72°C 10 minutes	72°C 7 minutes	60°c- 7 minutes	72°c-10 minute	72°c -5 minutes	72°c 10 minutes	72°C 10 minutes
Hold	Hold at 25°C	Hold at 25°c	Hold at 25°c	Hold at 25°c	Hold at 25°c	Hold at 25°c	Hold at 25°C

3) AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCT

After that for RNA isolation agarose gel electrophoresis was done. An agarose gel is prepared by combining 0.4 gm of agar in 40ml distilled water. It is heated very well by placing in a microwave oven until the agarose is dissolved. 1µl of ethidium bromide is added into it and swirled to mix. The gel should then be poured onto a taped plate with casting tools in position. Agarose should be poured without introducing air bubbles from the comb end to the other. Allow 20-30 minutes for solidification. Carefully the tape and the gel casting combs are removed and the gel was placed in a horizontal electrophoresis apparatus. IX TAE electrophoresis buffer was added to the reservoirs until the buffer just covers the agarose gel. At least one-tenth volume of 10X agarose gel loading dye to each DNA sample was then added. It was mixed in a paraffin strip, and loaded into the wells (2µl dye to 5 µl DNA sample). The lid of the electrophoresis unit was placed carefully the power pack Set enter 110 V (current should be less than 100mA) was then Switched on. After the required separation has been achieved, the power was switched off, take out the gel from the apparatus, and the lower parts were wiped out with tissue paper. Visualization of the DNA fragments on a long wave UV light in Gel Doc is done. After placing the gel in an exactly straight position the gel doc instrument is closed again. Now the option run protocol is pressed. The gel picture will be

displayed on the screen. From the image we could determine the presence of a viral gene by observing the DNA bands been produced.

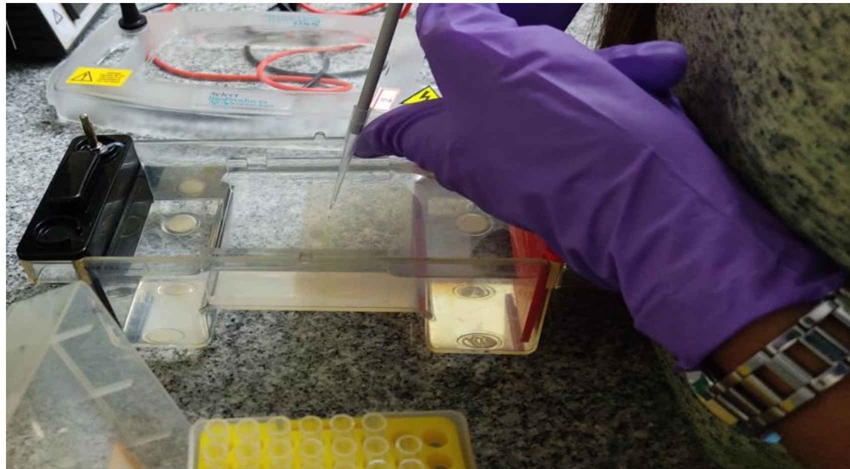


Figure 7: Preparation of Gel electrophoresis

VISUALIZATION OF DNA USING GEL DOC

As an initial step click on the option file on the screen of the computer that is been attached to the gel doc system. In the file click new protocol. Then click the 'select' option within the dialogue box. There appears another dialogue box in which press the option nucleic acids from which ethidium bromide is selected. Towards the end of the dialogue box there will be a highlight option and small tick near it. Remove the tick. Press on the option position gel. Since we have placed the gel in the gel doc now, we have to adjust its position in a straight manner since we could see the image of the gel on the computer screen by pressing on the position gel option. By this method all the viral tests for different viruses such as white spot syndrome virus, yellow head virus, taura syndrome virus, monodon baculovirus, hepatopancreatic parvovirus, early mortality syndrome, infectious myonecrosis virus, infectious hypodermal and hematopoietic necrosis virus were conducted.

Table 2: The methodology of each virology test being conducted on shrimps

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	Infectious hypodermal and hematopoietic necrosis virus (IHHNV)	PCR based detection *(Tang. et.al, 2000)*	Gills
4	Infectious Myonecrosis Virus (IMNV)	OIE manual of Diagnostic Test for Aquatic Animals. 2009. Chapter 2.2.3	Gills
5	Early mortality syndrome	PCR based detection (Han-Ching Wang. et.al, 2015)	Gills
6	Taura Syndrome Virus	PCR based detection Method	Gills
7	Penaeus monodon-type baculovirus Synonym MBV	PCR based detection Method (Lester et al., 1987)	Gills
8	Hepatopancreatic parvovirus disease (HPV)	PCR-based detection Method (khumsirichart et al., 1999)	Gills

TOTAL PLATE COUNT

Total Plate Count, also termed as total viable count, total mesophilic aerobic plate count, total bacterial count, etc. gives information about the number of aerobic bacteria present in a sample. A high bacterial count indicates the level of contamination of the product, conditions of storage, the extent of spoilage, etc. The TPC can be determined by the microscopic or culture methods. The cultural method is preferred for the determination of TPC because it gives an estimate of

viable (live) cells. In the procedure for sampling for TPC, a known quantity of the sample is macerated well with a known volume of a suitable diluent, and one ml of the appropriate dilutions are cultured in the plating medium.

For spread plate method, agar have to be poured and dried in advance. Melt two flasks of TGA in a water bath and then 1flask is cooled to 45°c. It should be poured into 6 Petri dishes, there should be allowed to set. The surface of the medium should be dried in 56°c incubator in a laminar air flow chamber for 45 minutes. Plates were cooled to room temperature. For sampling, first sterilise the glass mortar (interior) and the pestle by smearing with alcohol and flaming. Rinse with sterile NS. Aseptically 10g of skin with muscle of the given shrimp sample is cut and taken into a sample dish. It is Macerated with 90ml diluent (NS) in a sterile glass mortar (10' dilution). Pipette 1 ml of the supernatant to 9 ml diluent and mix well (10 dilution). For spread plating the pre-set TGA plates in 3 rows in duplicate were arranged and labelled appropriately. Inoculate 0.5ml each of the 10^{-1}, 10^{-2} and 10^{-3} dilutions on the surface of the respective agar plates. Spread the inoculum evenly on the surface of each dish with a sterile bent glass rod. Please note that between spreading operations of each plate, the glass rod should be sterilized by dipping it in alcohol and flaming it. After about 30 min. incubate the plates at 37°C (or room temperature) for 48 hrs. After 48 hours of incubation, the colonies developing in each plate are 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-dish.

TPC/g sample =Average count X dilution factor

*Dilution factor is reverse of the dilution, i.e., if dilution is 10, dilution factor will be 10 or 1000.

In the case of colony counts from spread plates, the average count has to be doubled before calculation of TPC since only 0.5 ml of the sample dilution was added in each plate.

BACTERIOLOGICAL TESTS

Bacteriology testing includes the cultivation of bacteria in specific growth conditions. The medium usually used to grow the bacteria is agar. A bacterial test is often conducted to determine any kind of bacterial infection in the organism. Agar is typically mixed with substances that stimulate bacteria to develop; a common example is sheep blood, which gives

nutrients to the bacteria as they expand. Bacteriological tests for the analysis of three different types of bacteria such as *Staphylococcus spp, Vibrio cholerae and E coli* were conducted in CMFRI by providing the samples to the microbiologists over there. The animals from each station were pooled and proceeded separately for microbial analysis. The whole animal along with gut was homogenized using sterile normal saline. Subsequently, 10-fold dilutions of each homogenate were prepared and each dilution was spread onto nutrient agar (salinity corresponded to the salinity of residing water of the animal), Thiosulfate- Citrate-Bile Salts-Sucrose Agar (TCBS agar), MacConkey agar, Eosin Methylene Blue (EMB) agar, Baird-Parker agar and Mannitol salt agar. All the plates except nutrient agar and TCBS were incubated at 37°C for 72 hours. Nutrient agar and TCBS were incubated at 30°C for 3 days. The colonies characteristic of targeted microbes was only taken into consideration during enumeration.

RESULT

WATER QUALITY PARAMETERS

SALINITY

The salinity of the water sample collected from Malipuram was estimated to be 7 ppt

The salinity of the water sample from Chellanam was 10 ppt.

DISSOLVED OXYGEN

Dissolved oxygen present in the water from Malipuram was recorded to be 11.2 Ppm

Dissolved oxygen in the water sample from Chellanam was recorded to be 10.4 Ppm

ALKALINITY

Within the water collected from Malipuram, the alkalinity was estimated to be 240 Ppm

Within the water sample collected from Chellanam, the salinity was estimated to be 235 Ppm

HARDNESS

The total hardness of the water sample from Malipuram was calculated to be 1632 Ppm

Total hardness of the water sample from Chellanam was calculated to be 1530 Ppm

TEMPERATURE

Temperature of the water sample collected from Malipuram was 28^0C

Temperature of the water sample collected from Chellanam was 29^0C

PH

PH of the water sample from Malipuram was measured to be 7.5

PH of the water sample from Chellanam was noted to be 7.7

Table 3: Water quality parameters

SL NO	Water quality parameters	Sample from Chellanam	Sample from Malipuram	Standard parameters
1	Salinity	10 ppt	7ppt	10- 20 ppt
2	Alkalinity	240ppm	235 ppm	75-150 ppt
3	Dissolved oxygen	10.4 ppm	11.2 ppm	More than 6
4	Hardness	1632 ppm	1530 ppm	Depends on salinity for 10 ppt salinity 3000 ppm
5	Temperature	29°c	28	25°- 35°C
6	Ph	7.7	7.5	7-8

SOIL QUALITY PARAMETERS

TEMPERATURE

The temperature of the soil sample from Malipuram was noted to be 30°c

The temperature of the soil sample from Chellanam was noted to be 28°c

PH

PH of the soil taken from Malipuram was recorded to be 8.3

PH of the soil taken from Chellanam was recorded to be 8.4

EH

Within the soil taken from Malipuram, the EH was measured to be -177

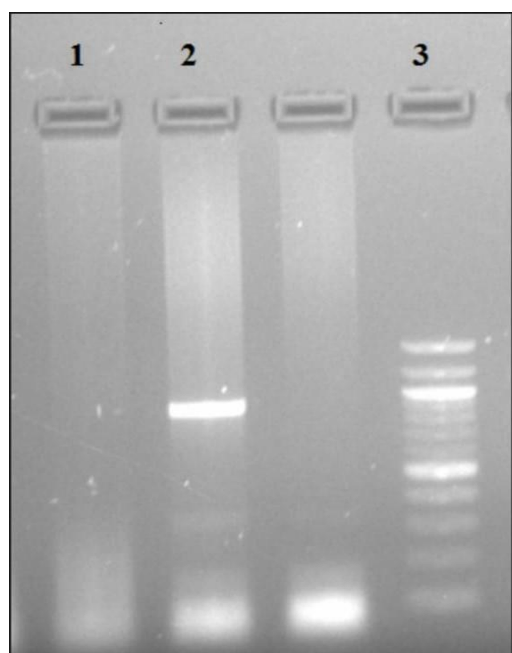
Within the soil taken from Chellanam, the EH was measured to be -175

Table 4: The soil quality parameters

SL NO	Soil quality parameters	Sample from Chellanam	Sample from Malipuram	Standard parameters
1	EH	-175	-177	-300 to 900
2	PH	8.4	8.3	6-9
3	Temperature	28°c	30°c	25°-35°c

VIROLOGY TESTS

According to the virological tests conducted in CUSAT, white spot syndrome virus was tested positive in the sample which was collected from Malipuram region. The detection of WSSV virus was done by the imaging of DNA done in Gel doc instrument after PCR technique and electrophoresis. The presence of virus was identified by comparing the width of DNA band with that of the bands which were formed from ladder. Bands of ladder was kept for comparison and for detection of diseases. Results indicated that a visible band at 941bp was appeared and it is as expected as positive against WSSV virus.



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

Reference: Lo et al, 1996, Disease of aquatic organisms

1. First PCR product

2. Second PCR product

Figure 8: Gel doc image of positive results against white spot syndrome virus

Figure 9: Negative results of virology test for IHHNV, HPV, TSV, YHV, MBV, IMNV, EMS

Table 5: The results of Virology tests

Sl. No	Parameter	Sample	Result
1	White Spot Syndrome Virus (WSSV)	Gills	**Positive**
2	Yellow Head Virus (YHV)	Gills	Negative
3	Infectious hypodermal and hematopoietic necrosis virus(IHHNV)	Gills	Negative
4	Infectious Myonecrosis Virus (IMNV)	Gills	Negative
5	Early mortality syndrome	Gills	Negative
6	Taura Syndrome Virus	Gills	Negative
7	Penaeus monodon-type baculovirus Synonym	Gills	Negative
8	Hepatopancreatic parvovirus disease (HPV)	Gills	Negative

BACTERIOLOGICAL TESTS

According to the bacteriological study report from CMFRI three different types of bacteria namely *E.coli, Vibrio cholerae and Staphylococcus aureus* were detected in shrimps collected from both Malipuram and Chellanam in variable amount.

Table 6: Results of bacteriological tests been conducted

Media/test	**Microbes targeted**	**Chellanam**	**Malipuram (CFU/g)**
TCBS agar	Presumptive vibrio's	$6.5* 10^2$	$1.3*10^3$
MacConkey agar	Presumptive coliforms	0	0
EMB agar	Presumptive *E coli*	0	0
MSA	Presumptive mannitol fermenting *Staphylococcus spp*.	$1* 10^3$	$4*10^3$
BP agar	Presumptive *Staphylococcus aureus*	$3.5*10^2$	$1.4*10^3$

TOTAL PLATE COUNT

Total Plate count test was conducted in order to determine the total rate of contamination and bacterial count within the specimen under study. There are two methods of total plate count they are either whole body method or gut method depending on the region of the body of specimen from where the sample for total plate count is taken . In the current study Total plate count test using whole body tissue was done in CIFNET and the same test using tissue from gut was carried out in CMFRI.

Table 7: Total plant count results

Media/test	Microbes targeted	Chellanam	Malipuram
Nutrient agar	Total viable count (gut)	$3.45*10^3$	$2.5*10^4$
Nutrient agar	Total viable count (whole body)	$9*10^3$	$45*10^3$

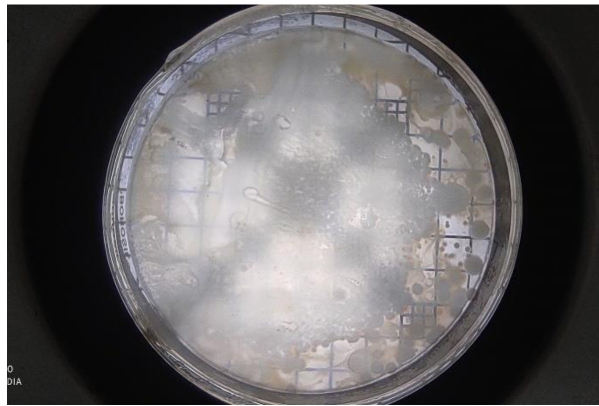


Figure 10 : Colonial growth of bacteria in the sample collected from Malipuram

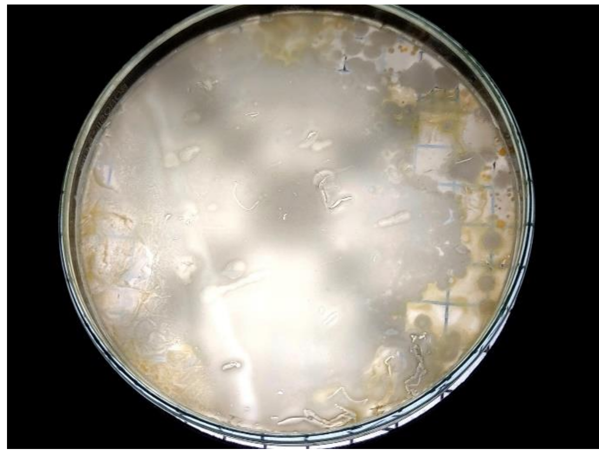


Figure 11: Bacterial colonies in the shrimp specimen been collected from Chellanam

DISCUSSION

Shrimp farming is an aquaculture organization that produces shrimp or prawns in either a marine or freshwater setting. Specific issues that may arise in shrimp farming commodities include contamination by pathogenic microbes such as *Salmonella* and *Vibrio cholerae,* as well as the presence of veterinarian medications (and other substances) that may be harmful to customers, workers, and the environment. In the current investigation, the risk factors and different types of pathogens that affect the growth and development of Tiger shrimps were studied in detail and both bacterial and viral presence in shrimp variety was assessed in an appropriate manner. All the physiochemical attributes of water and soil were conducted including pH, temperature, alkalinity, salinity, dissolved oxygen, hardness and eh were tested. Variations were observed in the values of Alkalinity, salinity and hardness of water sample been collected which can be an indication to the development of environmental stress within the natural habitats. The salinity from Malipuram farm was noted to be 7 ppt and from Chellanam was 10 ppt. Standard parameter values are recorded to be between 10 and 20 ppt which states that the salinity in Chellanam farm is normal and Malipuram farm is in declined condition. In compliance to the study conducted by Tendencia et al., (2011), the salinity, alkalinity, PH and microflora of the water from three different ponds were tested for different timings and the duration of study was between 2005-2006. In some of the observations, the salinity often dropped from 15 to 7 ppt. But the alkalinity rate was maintained equal to that of the standard parameters. There was also a deviation in the PH levels and temperature of the water from the standard parameters which can also be considered as an indication for the white spot syndrome virus been detected from the research (Tendencia et al., 2011). Acute salinity stress is more severe at low salinity than at high salinity, impacting P. monodon's immunocompetence and increasing vulnerability to WSSV infection (Joseph and Philip, 2007). Shrimps with low salinity have the lowest growth rate, which is related to energy expenditure for osmoregulation (Chen et al., 1994).

The adverse circumstances could have been caused by suboptimal temperature and salinity. The immune mechanisms of shrimps are influenced by temperature and salinity (Vargas-Albores et al., 1998; Le Moullac and Haffner, 2000). In the present study the temperature of water sample from both the regions are estimated to be 28°c and 29°c and is between the standardised ranges which are noted to be 25-30°c. But as per the studies conducted by

Tendencia et al., (2011) there is a fluctuation in the temperature approximately 3°c to 4°c from the standardised rates. The temperature was measured to be falling between 23°c and 33°c, presence of white spot syndrome virus along with vibrio species of bacteria was also detected in this study (Tendencia et al., 2011). Increasing temperature can disrupt crustacean equilibrium, resulting in metabolic stress and illness (Dove et al., 2010).

The variation in the rate of alkalinity was found to be much higher in comparison to the standard parameters. In Malipuram the alkalinity was noted to be 235ppm and in Chellanam it was 240 ppm whereas the standardized parameters were 75 to 150 ppm. In accordance to the research done by Nash et al., (1988) the alkalinity level of water collected from the ponds were much lower as it was diluted due to the extensive reduction in the pH. This work mainly focused on the pathogenesis caused by changes in alkalinity to *Penaeus monodon Fabricius* (Nash et al., 1988). The current study was also highly influenced by the variations in the hardness of water which was much lesser than normal values. In standardized conditions, the hardness is dependent on salinity that is if salinity is approximately 10 ppt then the hardness should be 3000 but in the current case even though the salinity in Chellanam was 10 ppt the hardness was estimated to be 1632ppm and in Malipuram it was 1530ppm. Depression in the values of hardness indicates the reduced presence of calcium and other elements. With respect to the studies performed by Latif et al., (1992) a reduced hardness level of water indicated an increased incidence of a pathological disorder referred to as "white muscle syndrome," a reduced size update achieved at moult while inevitably increasing moulting rate, and improved the accumulation of calcium in carapaces during intermoult (Latif et al., 1992). Through different studies it has been proven that any variations whether it can be an elevation or depression in the standard rates of physiochemical attributes can lead to an increased condition of pathogenicity due to the decrease in the immune resistance of shrimps.

Bacteriological tests for the analysis and detection of three different microbe varieties such as *E. coli, Staphylococcus aureus, and Vibrio cholerae* were done. As per the results of bacteriological tests it was been detected that Presumptive *E coli* and any other coliform bacteria were absent in tiger shrimps being collected from both Malipuram and Chellanam. As a part of the current work, total plate count was estimated. The sample from both the whole-body tissue and gut are collected and tested. The results showed that the total viable count of bacteria being collected from the gut as well as body tissue is approximately ten folds more in the specimen delivered from Malipuram when compared to Chellanam. The total plate count from Chellanam was reported to be 3.45*103 and 2.5*104 respectively through both methods

of Total plate method. This indicates that rate of contamination is more in the shrimps from Malipuram. Lekshmy et al., (2014), through their work determined that the rate of total plate count and *E coli* will remain the same throughout the three different seasons like pre-monsoon, monsoon and post-monsoon. In their study the rate of microbial growth in the extensive system was 4.1, 8.9, 6.7%; 95.5%, 88.1%, 92.6%, and 0.4, 2.9, 0.7%, respectively, for pre-monsoon, monsoon, and post-monsoon. TPC in water, silt, and shrimp in modified extended culture systems were 13.0, 7.2, 11.3%; 86.6%, 92.4%, 87.7%, and 0.4, 0.4, 1.0%, respectively, for pre-monsoon, monsoon, and post-monsoon. In water, the percentage spread of *E. coli* was greatest during the post-monsoon (11.6%) and lowest during the pre-monsoon (6.9%). During the three seasons, the percentage spread of *E. coli* in sediment and crustaceans was 88.5, 89.6, 85.1%, and 4.5, 2.9, 3.3%, respectively. The percentage spread of *E. coli* in water and crustaceans was determined in a modified extensive system over three seasons (Lekshmy et al.,2014).

The rate of *Staphylococcus aureus* present in the specimen collected from Chellanam and Malipuram are estimated to be 3.5*102 and 1.4*103 whereas the count of other species of staphylococcus species is detected to be 1* 103 and 4*103. According to (Yousuf et al., 2008), aerobic plate count (APC), *Enterobacteriaceae, and Salmonella-Shigella* (SS) counts were used to compare the frequency of microbial flora in the muscle of locally available tiger shrimp (*Penaeus monodon*) and giant water prawn (*Macrobrachium rosenbergii*). Overall numbers for shrimp ranged from 2.04x10 to 4.5x10 CFU/ml, while prawn counts ranged from 1.08x10 to 1.2x10 CFU/ml. For shrimp, the overall coliform count varied from 5.4x10 to 8.5x10 cells, and for prawns, it was between 5x10 and 4.4x10 cells. The overall counts for shrimp varied from 2.04x10 to 4.5x10 CFU/ml and for prawns from 1.08x10 to 1.2x10 CFU/ml. The total coliform count ranged from 5.4x10 to 8.5x10 cells for shrimp and from 5x10 to 4.4x10 cells for prawn. Furthermore, the *Salmonella-Shigella* (SS) count for the shrimp varied from 0.2x10 to 1.1x10 cells and for the crab from 0.26x10 to 0.96x10 cells. Sixteen isolates were identified from all samples on plate count agar, with the following percentages of various bacteria identified: *Staphylococcus aureus* (6.25%), *Salmonella sp.* (25%), *Shigella sp.* (12.5%), *Flavobacterium sp.* (12.5%), and *Vibrio sp.* (43.75%). The results showed that the microbial burden was greater in samples obtained from retail stores without appropriate treatment (Yousuf et al., 2008).

Out of the eight different virological tests been conducted white spot syndrome virus was detected to be positive. The major reason for the occurrence of disease may be due to the contamination with the water that flows from the other farms which are already affected. This was proven by the conduction of a survey study among the farmers. Through the surveillance

study it was determined that white spot syndrome virus have been tested positive in tiger shrimps found within some of the farms found in nearby regions of Malipuram, located within Vypin. Among all diseases, the white spot syndrome virus (WSSV) is by far an extremely lethal pathogen of cultivated crustaceans. It has the ability to infect all cultured penaeids and has been responsible for much of the economic impact on a global level (Thitamadee et al., 2016).

WSSV has been found in a variety of natural invertebrates, including crabs, lobsters, and shrimp, as well as hatchery-reared post-larvae from Asia (Fabricius, 1798). The WSSV virus is one of the most lethal viruses in shrimp cultivation, affecting penaeid shrimp. White spot viral illness has claimed many lives and severely harmed India's shrimp cultivation business. This virus affects all life phases of *P. monodon*, and the mortality rate can approach 100% within 3-9 days of clinical symptoms appearing (Ramos et al.,2014).

According to the study conducted by Tendencia et al., (2011), white spot syndrome virus is one of the most important and dangerous viral disease of shrimp which can lead to large scale mortality of shrimps. This research reveals the risk factors linked with two distinct farming techniques for *P. monodon*: polyculture and semi-intensive monoculture. Information was collected using an organized questionnaire. Data were gathered from 174 shrimp producers across eight regions in the Philippines. Forty-seven characteristics related to pond history and site description, method and period of culture, techniques for pond preparation, management of water, feed and other inputs, and biosecurity measures were investigated. Other than feeding live molluscs and sharing water resources with other farms, factors such as larger pond size and higher stocking density were identified as important WSSV risk factors in monoculture farms in the analysis for combined monoculture and polyculture farms (Tendencia et al., 2011).

Water quality was also considered as one of the important factors which influences the growth and culturing of shrimps. This study identified several unique factors that influence WSSV infection related to their feed, such as feeding live molluscs, which can lead to an increased risk, and culturing in a mangrove to pond area, which can reduce the risk. This research speculates that pond location and management influence the occurrence of WSSV. This research will use an epidemiological method to find these risk variables using a structured questionnaire (Tendencia et al., 2011). The current study has great similarities with the study conducted by Tendencia in several manners. In both situations the quality parameters were considered as a substantial element which determines the rate of growth, occurrence of disease and development of shrimps where the specimen under study was *Penaeus monodon*. The

studies had proven a fact that interconnections between the water resources can be the main reason for the contamination and cause of the diseases in shrimps. This fact was determined by a structured questionnaire been conducted among the local farmers who cultures shrimps.

The most effective way to prevent and handle viral infections is to practice sanitary practices in breeding facilities, nurseries, and ponds. Another method is the application and enhancement of stocks with high immune rate. Although shrimp have a primitive immune system, vaccination may be a helpful technique for potential viral illness protection. Treatment for prawn disease is difficult; frequently, it is more involved than illness prevention. There isn't one remedy for every issue, but there are precautions that can be taken to prevent viruses from invading shrimp ponds and to keep shrimp healthy. The FAO suggests certain recommended practices for breeding prawns.

Select prawn genotypes that are excellent performers and specific pathogen-free (SPF) for systems with high levels of biosecurity. Clean brood stock that provides eggs and larvae for hatcheries are the first step in improving biosecurity in prawn farming. Regardless of whether it is SPF or not, the brood stock should be properly acquired and verified. As it may stop the disease carriers from entering the culture system, this is the first and most crucial action to do. Maintaining regular facility drying, disinfecting fill and exchange water, regulating feed, and avoiding air and droplet contamination are all ways to keep infections out. Putting biosecurity into practise Reduce the number of visits as much as possible, including cars, people, birds, and other carriers (Shinn et al., 2018).

CONCLUSION

Shrimp farming is found to be a major technique that can help to improve the environment by reducing deforestation and by promoting sustainable practices. Pathogen introduction can cause massive epidemics of illness in shrimp, with disastrous effects. The purpose of the current study was to assess the bacterial and virological content within the *Penaeus monodon* variety of shrimp being cultured in both Chellanam and Malipuram areas. They were subjected to virological tests and bacteriological tests for the assessment of viral and microbial content within them. Along with the pathological analysis, the physiochemical parameters of soil and water collected from the natural habitats of specimen were also conducted to determine the quality of the resources in which the organism survive. The results of the analysis suggested that there are certain slight variations in hardness, and alkalinity of water from both the farm areas indicating the formation of an environmental stress within the habitats in which the shrimp survive. And a declined value of salinity was estimated from the water sample in Malipuram which can be a reason for the diseases or even a reduced growth rate in shrimps. Virological studies were conducted for the analysis of eight different viruses such as yellow head virus, taura syndrome virus, early mortality syndrome, infectious myonecrosis, infectious hypodermal and hematopoietic necrosis virus, hepatopancreatic parvovirus, monodon baculovirus, etc among which white spot syndrome virus was tested positive. From this result it was understood that even in the absence of symptoms the specimen can be infected with any kind of virus since the specimen under study never showed any symptoms that are commonly found in affected specimen. Bacteriological tests for the detection of *E coli, Salmonella and Vibrio cholerae* were outsourced from CMFRI which determined the absence of *E coli* bacteria in any of the specimen from both the areas. As per the total viable count test conducted in both gut and whole-body tissue, it is stated that the water in Malipuram is almost ten folds contaminated than Chellanam. Through the detailed study it has been determined that most of the pathogenic infections occurs in the organism through water. This conclusion was made by experimental research as well as the survey conducted in farmers, which provided the information that some of the aquaculture farms in Njarackal region near to Malipuram were recently affected with white spot syndrome virus in tiger shrimps and as a result their production was recently stopped. So, by detecting the disease in specimen collected from Malipuram it is proved that the diseases can be spread by water. Many extensive studies can be conducted on the shrimp pathology and various types of pathogens affecting them along with environmental conditions to detect various unknown pathogenic infections.

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