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Prevalence and Relative Virulence Properties of Circulatory Phylotypes of White Spot Syndrome Virus in Crustaceans

PhD Thesis

DEPARTMENT OF MICROBIOLOGY
UNIVERSITY OF DHAKA
DHAKA-1000
February 2022

SUBMITTED BY
REGISTRATION NO. **49**
SESSION: **2021-2022**

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A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN THE FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Dedicated to...

My Beloved Parents, Wife and Daughter Who Treasured My Life with Their Love and Affection

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Certification

It is hereby certified that student bearing Reg. No. 49, Session 2021-2022 has carried out the research work entitled "Prevalence and Relative Virulence Properties of Circulatory Phylotypes of White Spot Syndrome Virus in Crustaceans" for the fulfillment of his PhD Degree from University of Dhaka, Bangladesh, under our academic supervision and co-supervision in the Department of Microbiology, University of Dhaka.

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Abstract

White Spot Syndrome Virus (WSSV) is recognised as a highly prevalent and deadly virus worldwide which affects both shrimps and crabs in the coastal aquatic environment. This research work intended to observe the presence of deadly WSSV in selected ghers during the period of 2017 to 2019 and the virulence of WSSV's phylotypes circulating in Bangladesh. We had collected 360 shrimp (*Penaeus monodon*) and 120 crab (*Scylla* sp.) samples from the South-East (Cox's Bazar) and South-West (Satkhira) coastal regions of Bangladesh during monsoon, and the sampling was continued during post-monsoon also with the observation of same number of samples. PCR assays targeting VP28 gene uncovered statistically significant ($p < 0.05$, Kruskal Wallis test) differences in the WSSV's prevalence in shrimps and crabs of the selected regions of this study (Cox's Bazar and Satkhira), and also over the periods of this study (2017-2019). The mean Log copy numbers of WSSV varied from 8.40 (in Cox's Bazar) to 10.48 (in Satkhira) per gram tissue indicating serious diseased condition. In the ghers where WSSV-positive crustacean samples were found, the mean values for salinity, dissolved oxygen, temperature and pH were 14.71±0.76 ppt, 3.7±0.1 ppm, 34.11±0.38 ˚C and 8.23±0.38, respectively. Phylogenetic analysis based on the envelope protein VP28 gene exposed a substitution in the amino acid (aa) sequences, (Glutamic acid→Glycine) at 167^{th} position in the isolates of Cox's Bazar (referred to as phylotype BD2) in comparison to the circulating one (BD1) worldwide. In the experimental infection assays, shrimp post larvae (PL) challenged with BD1 and BD2 phylotypes with filtrates of tissue containing 0.423 X 10^9 copies of WSSV per mL ensured a median LT50 value of 73 hrs and 75 hrs, respectively. The experimental infection assay demonstrated higher mean Log WSSV copies (6.47±2.07 per mg tissue) in shrimp PL challenged with BD1 compared to BD2 (4.75±0.35 per mg tissue). Crabs that were challenged with BD1 and BD2 through ingestion method demonstrated 100%

mortality within 48 hrs and 62 hrs post-infection, respectively, having mean Log WSSV copies of 12.06±0.48 and 9.95±0.37 per gram tissue, respectively. Furthermore, expression of shrimp antimicrobial peptides (AMPs) penaeidin and lysozyme was at a low level in BD1 treated shrimp PL in comparison to BD2 treated shrimp PL. These results all together exhibited that relative virulence properties of WSSV based on the rate of mortality, viral load and expression of host immune genes in crustaceans could be affected by single aa substitution in VP28. Assessment of prevalence with a larger number of samples covering all the shrimp farming regions of Bangladesh in future along with gene expression profiling of all important immune genes of crustaceans infected with the circulating groups of WSSV can add values to get into the insights of the occurrence of WSSV infections.

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	Glutamic Acid to Glycine at 167th amino acid position signifies gaining flexibility using DynaMut web server. 'Blue' signifies a rigidification of the structure (G167E) (Figure 29A) and 'Red' represents a gain in flexibility (E167G) (Figure 29B).	

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Abbreviations

WSD	**White Spot Disease**
WSSV	**White Spot Syndrome Virus**
aa	**Amino Acid**
PCR	**Polymerase Chain Reaction**
UPR	**Unfolded Protein Response**
PL	**Post Larvae**
Ha	**Hectare**
hrs	**Hours**
HPI	**Hours Post Infection**
AMP	**Anti-Microbial Peptide**
C_T	**Threshold Cycle**
LT	**Lethal time**
Pen	**Penaeidin**
Lyso	**Lysozyme**
DNA	**Deoxyribonucleic acid**
cDNA	**Complimentary DNA**
RNA	**Ribonucleic acid**
dsDNA	**Double-stranded DNA**
dsRNA	**Double-stranded RNA**
NCBI	**National Center for Biotechnology Information**
MRCA	**Most Recent Common Ancestor**

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Abbreviated names of amino acids

G - Glycine
V - Valine
L - Leucine
I - Isoleucine
F - Phenylalanine
P - Proline
Y - Tyrosine
W - Tryptophan
S - Serine
T - Threonine
A - Alanine
M - Methionine
N - Asparagine
Q - Glutamine
D – Aspartic acid
E – Glutamic acid
K - Lysine
R - Arginine
C - Cysteine
H - Histidine

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Chapter 01:

Introduction and Literature Review

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1.1 General Introduction

White Spot Syndrome Virus (WSSV) is a serious viral pathogen which affects shrimps, crabs and other crustaceans and can transmit horizontally (Lo and Cou, 1998) and vertically (Lo et al., 1997). WSSV is spread quickly to farming areas and infects cultured shrimps and crabs very fast by its serious pathogenic characteristics (Balasubramanian et al., 2008). Shrimp aquaculture is a key earning source in Bangladesh along with many countries playing an extensive role for enlightening the advancement of community, food security, creating employment opportunities and poverty alleviation (Hossain et al., 2015) (Figure 1).

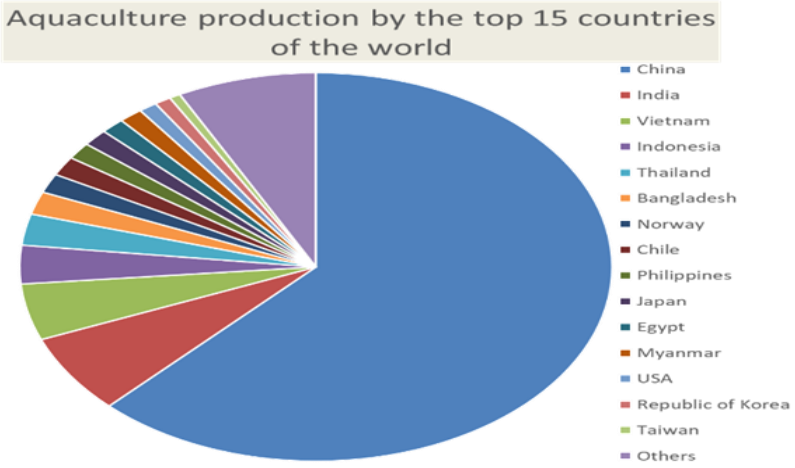

Figure 1.1 Leading countries in aquaculture production (Retrieved from www.fao.org accessed on 1 September 2019)

Figure 1.1 shows the top 15 countries of the world in aquaculture production. Shrimp aquaculture was reported to be among the fastest developing aquaculture industries (FAO, 2005). Rapid expansion and advancement of shrimp aquaculture in the world and in Bangladesh is deeply affected by the introduction of various disorders, particularly viral disease that jeopardizes the expansion of the industry. Shrimps, prawns and crabs play a vital

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role in culture-based production in the world as well as in Bangladesh. *Penaeus monodon* contributed 26% of the total fish production while the mud crab (*Scylla olivacea*) found in Bangladesh contributed 6% according to DoF, GoB. Freshwater prawns contributed 19% of the total production. There are some other shrimp species like *Metapenaeus monoceros* and *Fenneropenaeus indicus* which also contribute a little to the total aquaculture production in Bangladesh (Figure 1.2). Shrimp farming regions of Bangladesh include Khulna, Satkhira, Bagherhat, Barisal, Pirojpur, Patuakhali and Cox's Bazar districts. Farming areas in these districts are shown in Figure 1.2.

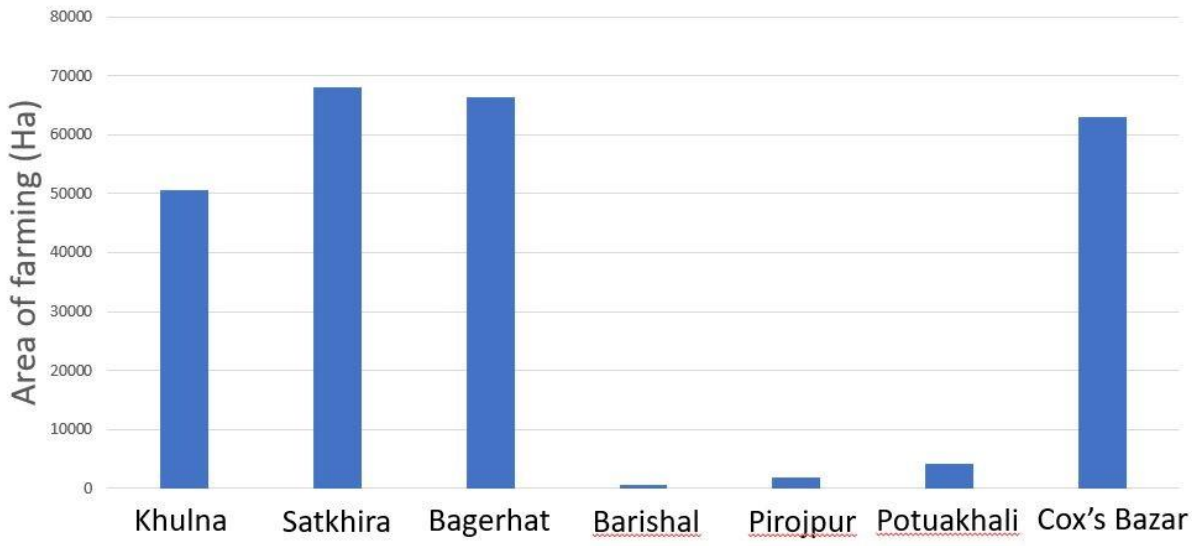

Figure 1.2 Shrimp farming areas (Ha) in Bangladesh (FRSS, 2017)

Shrimp culture being crucial to the economy of many countries gets affected by this serious viral pathogen (Lightner & Redman, 1998). The people of coastal zone of different countries completely depend on shrimp and crab culture. When this virus affects, people dependent on aquaculture must look for alternative income generating activities which lead to socioeconomic unrests impacting on the sustainable shrimp farming (Paul and Vogl, 2012). Figure 1.3 has been redrawn from Sohel and Ullah (2012) which shows the cause-effect

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relationship of shrimp farming in Bangladesh. The economic cost of White Spot Disease (WSD) on the shrimp farming business was assessed up to US$15 billion as it initially emerged growing at the rate of US$1 billion yearly (Stentiford et al., 2012). During the last 44 years, shrimp aquaculture had developed to be the utmost profitable division in aquaculture industries worldwide (Zhan et al., 2016). Penaeid shrimps and crabs like *Scylla* spp. are cultured in ghers in which the water enters from tidal water. Post-larvae of shrimps are collected from shrimp hatcheries where mother shrimps collected from the sea are used in hatching. WSSV can enter the shrimp ghers through these hatched larvae since WSSV can be transmitted vertically. Crustaceans can also be affected by WSSV horizontally by eating dead infected organisms or by the presence of WSSV infected organisms in the water body, artificial food and contaminated water of the gher. It is thought that physico-chemical properties may have important impacts on transmission of WSSV and infection by this deadly virus (Tendencia and Verreth, 2011). WSSV has a broad range of potential hosts that include not only the penaeid shrimps but also decapods like crabs, lobsters and crayfishes (Lo et al., 2005). Crabs with a low viral load (Somboonna et al., 2010) is not diseased but can be a very important reservoir of WSSV that can transmit horizontally to shrimps and cause disease when cultured together.

The worldwide production of shrimp was 4200 metric tonnes, appreciated at USD 4.8 billion in 2016 (GAA, 2017) which is in huge risk due to diseases (FAO, 2012). Rahman and Hossain (2013) mentioned in their study that 97% of the shrimps produced were exported which could contribute about 5% to the national economy of Bangladesh being the second most foreign currency-earning source.

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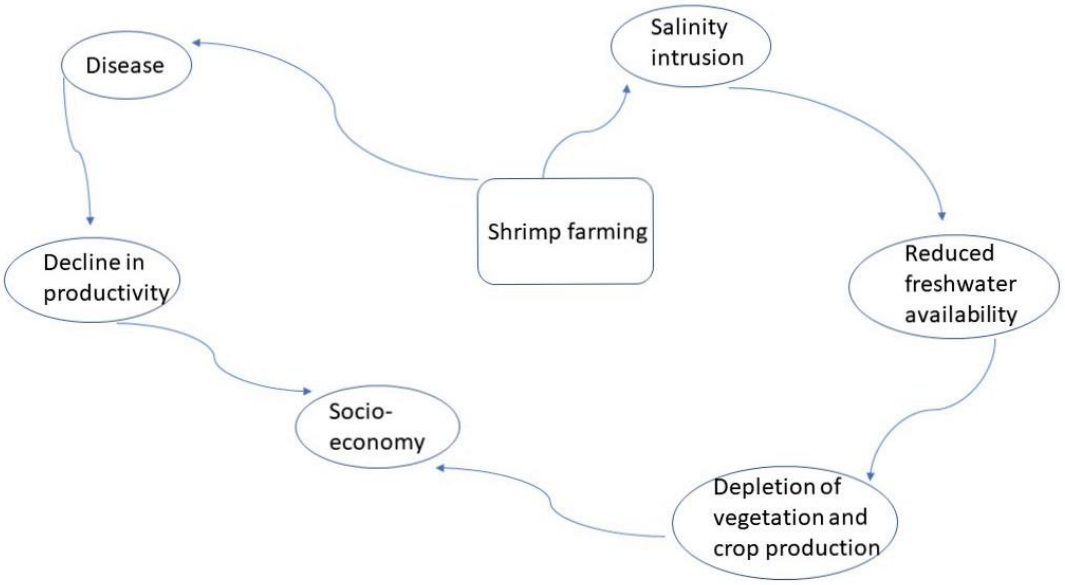

Figure 1.3 The cause-effect relationship of shrimp farming in Bangladesh (Redrawn from Sohel and Ullah (2012))

Approximately 85 million people of Bangladesh, mostly of coastal regions, for their livelihood, depend on this socio-economically important sector (DoF, 2013). WSSV, being responsible for White Spot Disease (WSD), causes massive economic deficits in the coastal areas of Bangladesh every year (Paul and Vogl, 2012). 3 to 10 days of infection by WSSV causes up to 100% mortality of shrimps resulting in such massive losses to the industry for WSSV's capacity of fast reproduction and enormous virulence (Kimura et al., 2000; Arturo, 2010; Zhu et al., 2019). Several crustacean species in natural, man-made, and experimental settings can be infected by WSSV leading to a complete mortality of shrimps in the facility within a week (Zhu et al., 2019; Vaseeharan et al., 2003). *Penaeus monodon*, the black tiger shrimp, constitutes the second largest export commodity of Bangladesh playing a very significant role to the national economy (Mazumder et al., 2015). Proneness of *Penaeus monodon* towards lethal viral diseases remains continuously an upsetting feature in

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aquaculture business, and WSSV which is a rod-shaped dsDNA virus of *Whispovirus* genus (305.12 Kb length) is the most lethal (Chakrabarty et al., 2015). Black tiger shrimp and crabs contribute 26% and 6%, respectively, to the total aquaculture production in Bangladesh (FRSS, 2017) (Figure 1.4). The mud crab (*Scylla* spp.) farming is showing a trend of increasing in the coastal regions of Bangladesh for being comparatively less prone to diseases and their high prices in the market. This type of farming supported livelihoods of many people involved in crab fishery, trading and exporting (Molla et al., 2009).

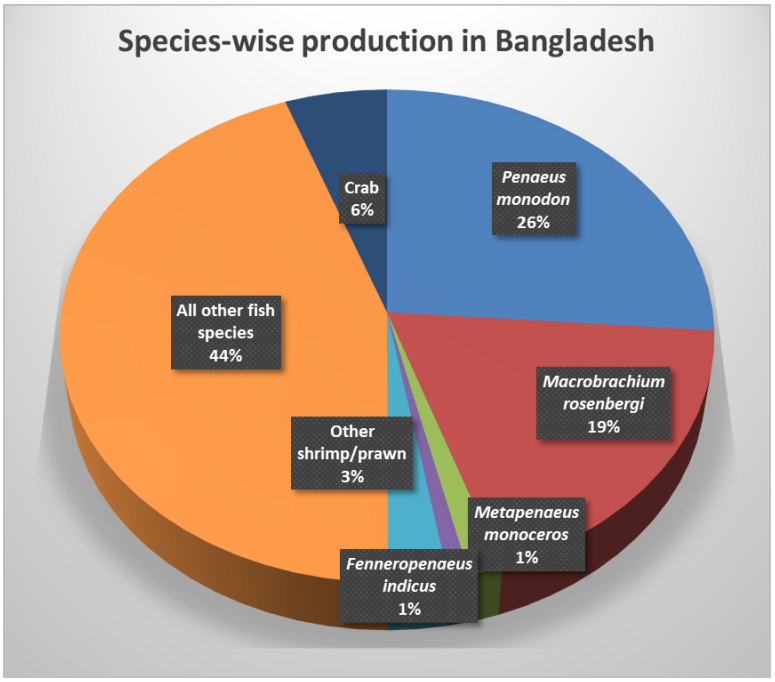

Figure 1.4 Species-wise production in Bangladesh (FRSS, 2017)

Crabs, relatively, are supposed to be at a lower risk to the impacts of the changing climate and degradation of water quality which are considered as threats as carriers of WSSV and for the ability to remain affected for extended periods without any symptoms of WSD (Flezel et al., 2006; Kanchanaphum et al., 1998; Supamattaya et al., 1998). WSD is regarded as the utmost hazardous and dreadful disease in shrimp and crab farms of Bangladesh which can

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lead to 100% mortality of the shrimps in culture ponds after the appearance of clinical signs (Chou et al., 1995; Yang et al. 1999). WSD was reported for the first time in shrimps from China and then distributed to the different geographical locations of the world (Chou et al., 1995). The causative agent WSSV has three layers over the double-stranded DNA; a nucleocapsid, a tegument and an envelope (Oakey and Smith, 2018). There are structural proteins found in the envelope that were reported to produce neutralizing antibodies and as targets to design vaccineagainst the most virulent pathogens of penaeid shrimp in the world (Momtaz et al., 2018). This large double-stranded and enveloped DNA virus which belongs to the Family *Nimaviridae* are responsible for the most devastating disease in crustacean aquaculture (Mayo, 2002).

Over the past two decades, WSSV played its role being a major threat to the world shrimp aquaculture. The WSSV with a 300-kb circular double-stranded DNA can infect a variety of decapods and non-decapods in aquatic environment (Sun et al., 2014; Xie et al. 2015) with a capability to transmit both horizontally and vertically (Sánchez-Paz, 2010; Xie et al., 2015). With an occurrence of a WSSV-outbreak, entire populations of crustaceans in numerous aquaculture farms were wiped out in only a few days leading to enormous economic losses (Zhu et al., 2019). Kwang (2011) also reported 100% mortality after WSSV infection in 7-10 days.

WSSV's circular genome is around 275 nm in length and 120 nm in width with tail-like appendage at one end, and was reported to contain five key structural proteins: VP28, VP26, VP24, VP19 and VP15 (Kwang, 2011; Tsai et al., 2004). Research works on WSSV's structural proteins have shown the association of VP28 and VP19 with the virion envelope (Sarathi et al., 2008; Tsai et al., 2004). Moreover, VP26 performs as a tegument protein

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linking the two nucleocapsid-associated proteins VP24 and VP15 to the virion envelope (Musthaq et al., 2009; Sun et al., 2014). Interactions between WSSV and its hosts including the environment of their habitat have become key focus of many research works in recent years as the outbreak of WSD has key associations to these interactions (Sun et al., 2014). The VP28 is a key element of viral envelope essential for the entrance of WSSV into the host cell by endocytosis, cell-to-cell infection and virus propagation (Sarathi et al., 2008). Moreover, a substantial part in the initiation of WSSV infection is also played by this envelop protein (Sarathi et al., 2008). The VP28, established as the potential target for dsRNA treatment in research works (Nilsen et al., 2017) has been reported to participate in WSSV's escape from endosome by the VP28's interaction with receptor protein Rab7 of the host (Verbruggen et al., 2016).

Recent studies reported that geographically distant strains of WSSV were similar and closely-related apart from small genetic differences (Li et al., 2017; Parrilla-Taylor et al., 2018). Earlier assessments of strain variability opined about the competitive fitness to depend on the genome size (Parrilla-Taylor et al., 2018; Zwart et al., 2010); however, those studies are still far away to illustrate the complete mechanism of WSSV pathogenesis. The ability of WSSV has also been observed to infect shrimps irrespective of age and sex (Peinado-Guevara and López-Meyer 2006) in all categories of culture systems including extensive, semi-intensive and intensive (Chakrabarty et al., 2015). Along with vertical transmission, the outbreak of WSD can also occur due to horizontal transmission or any career organism in culture pond water, environmental factors and lack of proper management (Hoa et al., 2011). Cells infected with WSSV were found in numerous tissues including the gills, stomach, cuticular epidermis and other tissues originated from mesoderm and ectoderm in the crustaceans that were fed WSSV-contaminated food (Chang et al., 1996). Various challenge studies with

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different methods of inoculation have demonstrated cells infected with WSSV in gills, foregut, integument and connective tissues of the hepatopancreas in *Penaeus monodon* juveniles through in-situ hybridization assays after 16 hours of inoculation (Escobedo-Bonilla et al., 2008). There are controversial outcomes creating debate on the entrance site of WSSV in hosts. Host epithelial cells of the midgut were observed to be WSSV's entrance path in *Penaeus japonicus* in another study where shrimps were inoculated through oral administration. The role of hemocytes in circulating WSSV in crustacean tissue is also not confirmed as there were different studies with different ideas regarding the matter (Escobedo-Bonilla et al., 2008). But it was confirmed later that WSSV spread in a cell-free form through the circulation of haemolymph (Escobedo-Bonilla et al., 2007) inside the body of infected crustaceans. There are white spots formed by many species found in America and Asia after WSSV infection, the exact mechanism is not confirmed yet. It was suggested that WSSV infection could stimulate abnormal functioning of the integument, ultimately leading to calcium accumulation in the cuticles (Wang et al., 1999).

Being very similar to other invertebrates, shrimps have innate immunity, which is the first-line protection mechanism for fighting against infection. Such resistance mechanism eliminates invasive microbes by the use of cellular and humoral innate immune retorts (Söderhäll and Cerenius, 1998). Effective approaches for analysing gene expression profiles of immune genes, real-time PCR and micro array were used for studying WSSV pathogenesis in response to infection caused by WSSV (Shekhar et al., 2015) which targeted to observe the expression of *Penaeidin* (PEN), *Lysozyme* (lyso), *Caspase* and *Rab7* genes connected to shrimps' immunity. *Penaeidin* (PEN) and *Lysozyme* (lyso) are most common anti-microbial peptides (AMPs) that abolish cell walls of microorganisms through hydrolysis (Destoumieux et al., 2000). *Caspase* is an apoptosis related gene. Apoptosis creates an interesting condition

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in the WSSV infected shrimp in which both shrimp and virus make use of apoptosis and anti-apoptosis type genes to prompt individual responses as protective mechanism against viral infection and increasing viral reproduction, respectively (Shekhar et al., 2015). *Rab7,* receptor protein of WSSV, participates in regulating processes of multiple trafficking which includes the biogenesis of the lysosome, phagosome, autophagosome, and other lysosome-related organelle (Feng et al., 2019). *Rab7* was observed to be associated with WSSV infection to *P. monodon* as a VP28-binding protein (Feng et al., 2019). Nevertheless, the detailed knowledge of the *Rab7* effectors in shrimp and its part in the transport of WSSV is scarce even now. A mechanism for WSSV's entrance into the host-cell and interactions with the cell that ultimately lead to death of the crustaceans has been redrawn from Verbruggen et al. (2016) depicted in Figure 1.5.

After entering the host cell, the proteins of WSSV interacts with the receptors of host leading to induction of the endocytosis. The WSSV then passes through endosome and pH decreases during maturation, and at this stage there might be a possible interaction between VP28 and Rab7. With an unknown process of passing through the nuclear envelope WSSV enters the nucleus where transcription factors of the host bind the genome of WSSV and the viral genes begin to express. WSSV halts the host cell cycle in the S-phase through E2F1 to use the cell machinery of the host for DNA replication of the virus. Pathways of UPR can be activated when it is called the ER stress after production of a high level of viral proteins. UPR's transcription factors can help in the expression viral genes which ultimately could turn into the inhibition of translation. Binding of iron to Ferritin, which is done by the host to withhold iron, is inhibited as WSSV requires iron as a nutrient for its replication (Verbruggen et al., 2016).

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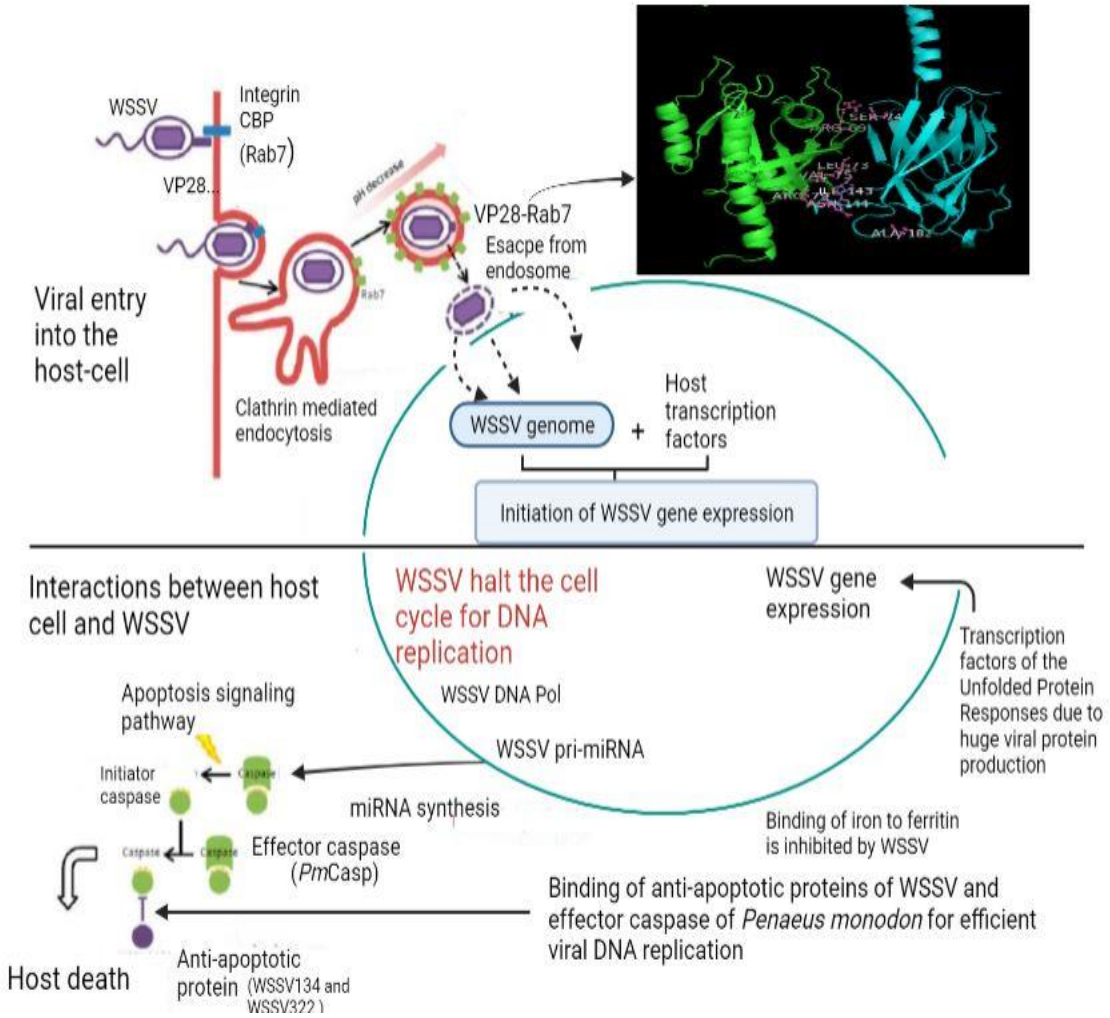

Figure 1.5 WSSV entry and interactions with host-cell (Verbruggen et al., 2016)

The apoptosis signaling is also influenced by WSSV either through the inhibition of activity of initiator caspases which is mediated by miRNA or using viral proteins which can inhibit the activity of effector caspases (Shekhar et al., 2015).

However, in culture ponds, appropriate protective management should be taken prior to stocking to the farm that are immensely crucial for lowering infections of WSSV in shrimp farms (Hossain et al., 2014). It is very important to monitor the biotic and abiotic factors of the culture ponds regularly after stocking healthy PL. Presence of pathogens leads to huge loss in the aquaculture industry. As Cox's Bazar and Satkhira, both districts are situated in

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different geographic locations inside the country, comparison of WSSV's occurrence through monitoring percentage presence and quantifying WSSV copies in tissue of affected crustaceans is crucial. It is also important to study regularly which kind of unfavorable conditions in the pond environment can lead to White Spot Disease. Siddique et al. (2018) identified two groups of WSSV from VP28 based phylogeny, one with Glutamic acid at amino acid position 167 of VP28 (BD1) and rest one showed mutation on that position with Glycine (BD2). The virulence properties of these phylotypes were of great importance to be determined which might help in reduction of the impacts of WSSV in shrimp farms.

1.1 Review of Literature

1.1.1 Diseases of shrimps

Shrimp diseases are the major limiting factors of aquaculture production. Diseases risk the production of shrimps and any other crustaceans like crabs present in the ghers. Usually in the improved traditional ghers of Bangladesh, mud crabs are also cultured along with the black tiger shrimps. Among all types of diseases, viral, bacterial, fungal and parasitic diseases are reported to be infectious. Viral diseases are caused primarily by DNA viruses and RNA viruses. Families of viruses pathogenic to penaeid shrimps are Parvoviridae, Baculoviridae, Iridoviridae and Nimaviridae. Pathogens of shrimps in Parvoviridae are Lymphoidal parvo-like virus (LPV), Spawner-isolated mortality virus (SMV), Hepatopancreatic parvovirus (HPV) and Infectious hypodermal and hematopoeitic necrosis virus (IHHNV). Pathogens of Baculoviridae include Hemocyte infecting non-occluded baculo-like virus, Type C baculovirus, Baculovirus midgut gland necrosis virus (BMNV), Monodon baculovirus (MBV) and Baculovirus penaei (BP). Shrimp iridovirus (IRIDO) and White spot syndrome virus (WSSV) are the only pathogens from the families Iridoviridae and Nimaviridae, respectively. There are seven classified pathogenic RNA virus families which include

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Picornaviridae, Roniviridae, Reoviridae, Rhabdoviridae, Togaviridae, Totiviridae and Bunyaviridae. Taura syndrome virus, Reo-like virus (REO), Mourilyan virus (MOV), Lymphoid organ vacuolization virus (LOVV), Infectious myonecrosis virus (IMNV) and Rhabdovirus of penaeid shrimp (RPS) are the single pathogenic viruses of such crustaceans which belong to the familes Picornaviridae, Reoviridae, Bunyaviridae, Togaviridae, Totiviridae and Rhabdoviridae. Roniviridae is known to have three pathogenic viruses of shrimps which are Lymphoid organ virus (LOV), Gill associated virus (GAV) and Yellow head virus (YHV). The overview of these major important pathogens and their families have been reviewed from the studies of Lightner (1996), Chang et al. (1993), Lightner and Redman (1998), Tang et al. (2005), Tang et al. (2007), Mayo (2002) and Sritunyalucksana et al. (2006).

Bacterial diseases can also be infectious and opportunistic. Bacteria has the capacity to create disease in crustaceans in any unfavorable environmental condition also. Appropriate protective management should be taken to prevent bacterial diseases in shrimp farms. However, the most reported key bacterial diseases of shrimps are mycobacteriosis, rickettsial infection, shell disease, hepatopancreatitis, filamentous bacterial disease and vibriosis (Lightner, 1996; Jayasree et al., 2006; Horowitz and Horowitz, 2001).

Fungal diseases are also infectious and can occur during larval stage while in the shrimp hatcheries and also during grow-out (Lightner, 1996). Parasitic diseases are caused by mainly haplosporidian, microsporidian, annelid worms, flagellates, ciliates and finally protozoans present in the water body (Lightner, 1996; Gopalakrishnan and Parida, 2005).

1.2.2 White Spot Disease

WSSV, the etiological agent of WSD, causes huge destruction in shrimp aquaculture resulting in massive economic losses (Paul and Vogl, 2012). WSD is very often recognised by white spots in the exoskeleton and epidermis of the shrimp (Figure 1.6). Along with the presence of white spots on shells, loose cuticles and discoloration of the integument are major clinical symptoms of WSD. This disease reflects the greatest upsetting ailment in shrimp culture in Bangladesh leading to 100% death in 3–10 days after the appearance of the clinical symptoms (Chou et al., 1995; Wang et al., 1998), causing huge losses in Asia posing greatest risk to the crustacean aquaculture business (Flegel, 2006). However, this disease had been gradually growing since 2007 in Bangladesh (DoF, 2013).

Diseases of shrimps and crabs in culture ponds are the most important factors controlling the production in shrimp farms as the crustaceans in culture ponds suffer from both infectious and non-infectious diseases (Lightner and Redman, 1998). There are important factors like physico-chemical parameters and biological components that may take part in playing a vital part in the pond ecosystem and the introduction of diseases. It is important to control viruses, bacteria, fungi and parasites in the ponds along with the physico-chemical properties through proper management and aquaculture practices (Horowitz and Horowitz, 2001).

Figure 1.6 Symptoms of White Spot Disease (Source: Samples of this study)

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1.2.2.1 Prevention

Previous investigations are there to look for whether preventative measures could be taken by increasing the immunity of the crustacean by feeding agents with pathogen-associated molecular patterns (PAMP) known for the activation of host innate immunity. *P. japonicus* were fed peptidoglycans and significant decrease in mortality was observed after challenge with WSSV. Injection of β-glucan also demonstrated to reduce mortality. Since there is lack of an adaptive immune system in the invertebrates, development of vaccine in traditional ways was not possible for such innate immunity-dependent organisms. "Immune priming", for example, rapid safeguard against WSSV can be attained for a short-term through exposing shrimps to inactivated viral particles. Inactivated virus, recombinant viral proteins, double-stranded RNA and viral DNA have given short-term protection (not more than 14 days) against WSSV (Mushtaq et al., 2009). However, *Bacillus subtilis* and baculovirus spores were modified and used to express VP28 to prepare oral vaccine (Mushtaq et al., 2009). It was also thought that RNAi could be used in the interactions between host and pathogen. It was observed in shrimps that small RNA molecules of host targeted and inhibited the expression of viral proteins. *M. japonicus* was observed to produce small interfering RNA (siRNA) which targeted VP28 when infected by WSSV. It was also found that blocking synthesis of siRNA caused increased number of viral copies in the infected organisms indicating protective role of RNAi (Huang & Zhang, 2013).

Biosecurity should be practiced in culture ponds of crustaceans to prevent WSD (Menasveta, 2002; Lightner, 2005) by introducing SPF shrimp stock, good quality feed, exclusion of pathogenic microorganisms from brood stock, treating water prior to fill in grow out ponds. Maintaining workers' hygiene are also of greatest importance to counter WSSV (Lightner,

2005). Although biosecurity measures are widely practiced nowadays in farms, these are not absolute (Schuur, 2003). Proper management to remove dead crustaceans from farm, maintaining a better feed management may reduce the danger.

1.2.2.2 Impact of physico-chemical parameters

WSD may increase in shrimp ghers when shrimps get exposed to different environmental stresses because factors creating stresses compromise the immune system of crustaceans and multiply quickly inside shrimps to cause mortality (Takahashi et al., 1995; Lo and Kou, 1998; Doan et al., 2009). Corsin et al, (2001) mentioned in their study that high pH in the water of shrimp culture ponds could be a risk factor associated with WSSV outbreak. It is reported that due to heavy rains, temperature and salinity fluctuations may occur which may further create stress for shrimps leading to WSSV infection causing mortality by rapidly increased viral loads in the shrimp body (Peinado-Guevara and Lopez-Meyer, 2006). Karunasagar et al. (1997) reported that there was occurrence of WSSV outbreak with a decrease in salinity in the west coast of India. Hettiarachchi et al. (1999) also confirmed low salinity to be a risk factor leading to WSD in shrimp ghers. Liu et al. (2006) mentioned in their study that acute salinity changes along with small adjustments in salinity may lead to increased WSSV replication. Salinity below 15 ppt and 3-4 C temperature fluctuations along with high *Vibrio* counts could be crucial risk factors associated with WSSV outbreaks (Tendencia and Verreth, 2011) and did not show any impact of dissolved oxygen making shrimps susceptible to WSD.

1.2.3 White Spot Syndrome Virus (WSSV)

Emergence of a new virus in shrimp farms of Taiwan was observed in 1992. Later in Japan it was isolated in late 1993 and was found to spread in different shrimp farming regions of the world. It was primarily thought that not a single virus was involved in spreading to different regions and was given different names which was later identified as a single viral agent

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involved in such infections of crustaceans (Escobedo-Bonilla et al., 2005). This virus named White Spot Syndrome Virus (WSSV) is a bacilliform and nonoccluded enveloped virus ranging in length between 210 and 380 nm and 70-167 nm in width (Figure 1.7). Electron micrographs with negative staining show tail-like appendage at an end sometimes. The viral envelop is a lipidic and trilaminar structure with a nucleocapsid having a stacked ring structure. The thickness of the envelop is 6-7 nm. Length of the nucleocapsid increases with the release from the envelop which indicates that it is tightly packed inside envelop. The length of the nucleocapsid ranges from 180 to 240 nm and width from 54-85 nm with a thick wall of 6 nm. This nucleocapsid contains the viral DNA. VP28, along with VP68, VP281, VP466 and even VP24 were found to have a role in penetration through in vivo neutralization assays. A cell attachment motif with a job in the entrance of virus is also present in envelope proteins VP31, VP110 and VP281 (Huang et al., 2002a; Tsai et al., 2004; Li et al., 2005; Xie et al., 2006), tegument protein VP36A and also the nucleocapsid proteins VP664 (Tsai et al., 2004; Leu et al., 2005) and VP136A (Tsai et al., 2004; Xie et al., 2006). Table 1 contains information about the whole genome sequences of WSSV published in NCBI Genbank database which had 91-97% homology in sequences. Oakey and Smith (2018) reported that WSSV contains 904 open reading frames and several homologous regions with partial repeats (Figure 1.8A-1.8B).

Size of genome sequences of WSSV varied from around 281 kbp to 312 kbp reported previously. Oakey and Smith (2018) reported genome sequence (WSSV-AU) comparatively smaller than the most others but within the range previously recognised. Marks et al. (2005) reported WSSV from Thailand, one of 293 kbp and one of 312 kbp. Infection assays with similar WSSV copies demonstrated WSSV with smaller genome size showed significantly quicker and higher rates of mortality.

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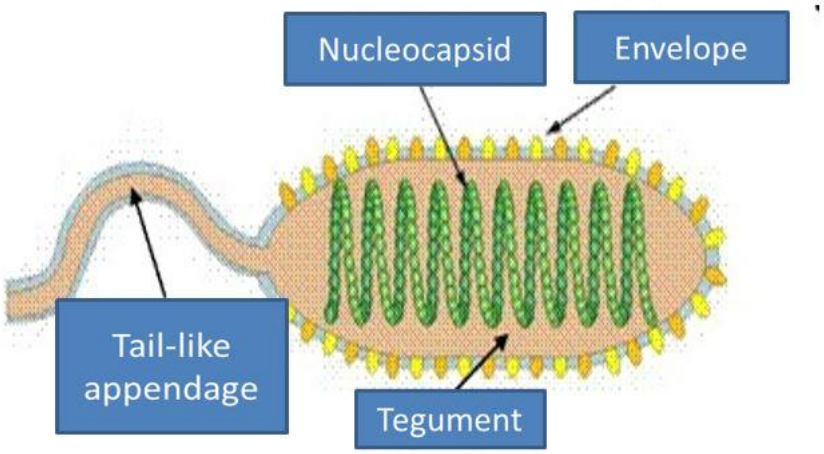

Figure 1.7 White Spot Syndrome Virus (Leu et al., 2009)

Across Asia, WSSV evolved to decrease its size by deletion of few genes which increased the genetic fitness of the virus and the pathogenicity after its emergence in 90s (Zwart et al., 2010), and the authors also proposed shorter survival of hosts when infected with WSSV. On the contrary, two genomes of WSSV (in 2010 and 2012) from China showed faster and higher mortality rates in shrimps challenged with those and suggested shorter genomes to be more virulent (Li et al., 2017).

For dealing with the primary obstacle, host's cell membrane, WSSV uses endocytosis process with interactions of proteins to enter the host's cell (Mercer et al., 2010; Huang et al., 2015).

VP 28, the major envelope protein, seems to be the key actor in the interactions with host protein (Kalia and Jameel, 2011). Verbruggen et al. (2016) in their review listed WSSV-host protein interactions. VP24, VP32, VP39B, VP41A, VP51B, VP53A, VP53B, VP60A, VP110, VP124 and VP337 have been found to interact with *Penaeus monodon*'s chitin-binding protein (PmCBP); VP53A with Glut1; VP28 with Rab7 (PmRab7); WSSV449 with Caspase (PmCaspase); WSSV134 and WSSV332 with Caspase (PmCasp); ICP11 with

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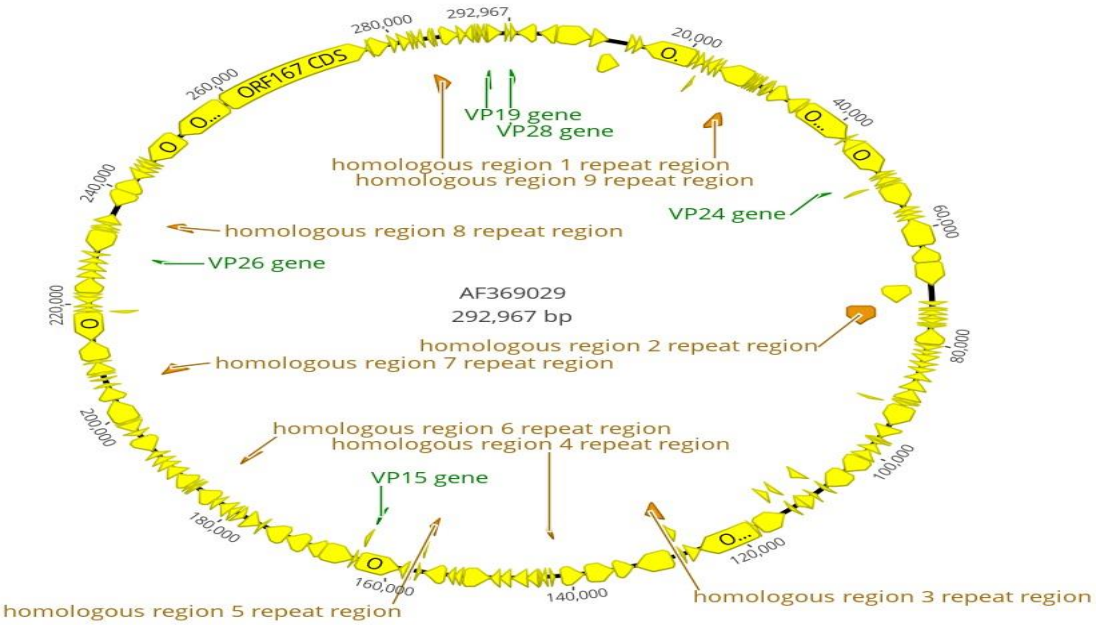
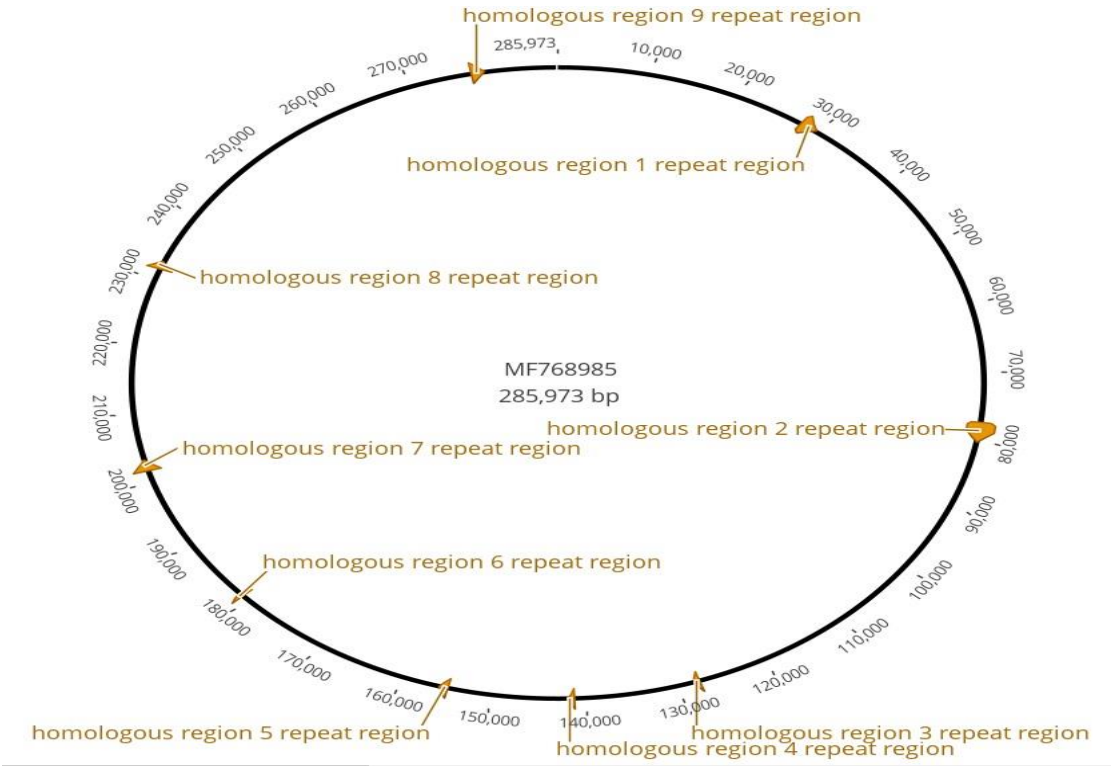
Table 1: Whole genome sequences of WSSV downloaded from NCBI

Accession no.	Country of origin	Year of last modification	Host	Genome size bp	GC content %	167th amino acid of VP28
AF332093 (Yang et al., 2001)	China	2014	*Penaeus japonicus*	305119	41	E (GAA)
JX515788 (Chai, 2013)	South Korea	2012	*Litopenaeus vannamei*	295884	40.9	E (GAA)
KT995470 (Fang et al., 2017)	China	2015	*Procambarus clarkii*	294261	41	E (GAA)
KT995471 (Fang et al., 2017)	China	2015	*Litopenaeus vannamei*	284148	41	E (GAA)
KT995472 (Fang et al., 2017)	China	2015	*Marsupenaeus japonicus*	309286	40.9	E (GAA)
KX686117 (Jiang et al., 2017)	China	2018	*Procambarus clarkii*	300223	41	E (GAA)
KY827813 (Li et al., 2015)	China	2017	*Marsupenaeus japonicus*	281054	41	E (GAA)
AF440570 (Tsai et al., 2000)	Taiwan	2005	*Procambarus clarkii*	307287	41	E (GAA)
KR083866 (Megahed et al., 2019)	Egypt	2019	*Fenneropenaeus indicus*	305119	41	E (GAA)
MH090824 (Restrepo et al., 2018)	Ecuador	2018	*Litopenaeus vannamei*	288997	41	E (GAA)
NC_003225 (Fang et al., 2015)	China	2018	*Marsupenaeus japonicus*	309286	40.9	E (GAA)
AF369029 (van Hulten et al., 2001)	Thailand	2005	*Penaeus monodon*	292967	41	E (GAA)
MF768985 (Oakey and Smith, 2018)	Australia	2017	*Penaeus monodon*	285973	41	E (GAA)
KU216744 (Rodriguez et al., 2016)	Mexico	2018	*Litopenaeus vannamei*	300087	41	E (GAA)
MG702567 (Katneni et al., 2018)	India	2018	*Penaeus vannamei*	280591	40.8	E (GAA)



A



B

Figure 1.8 A and B are circular overviews of the whole genomes of Taiwan and Australian WSSV generated by Geneious Prime Trial Version.

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Histones; VP9 with RACK1 (PmRACK1); VP15 with FKBP46 (PmFKBP46); and WSSVIE1 with TATA box-binding protein (PmTBP). During the escape from endosome in endocytosis, Rab7 has been found to bind with the major structural protein VP28. It may also be possible that this binding could occur after WSSV's escape from endosome (Mercer et al., 2010).

As long as the virus reaches the nucleus of the host, it needs to express its own genes which is only possible primarily by using the transcriptional machinery of the hosts (Qiu et al., 2014). For activation of transcription, transcription factors of the host need to bind with viral promoters (Liu et al., 2007). In Black Tiger Shrimp, viral gene *ie1* is involved in virus-host gene expression interactions being target of the host transcription factor STAT (PmSTAT) (Liu et al., 2007). The expressed genes at this stage encoding transcription factors are called 'immediate early genes', while the expression of genes dependent on these genes are called 'early genes'. 'Late genes' of WSSV are the genes expressed during the initiation of viral DNA synthesis and are included as structural proteins. *Ie1* has an element which play the role of initiator, called TATA-box, and also a site for binding with transcription factor PmSTAT (Liu et al., 2011). In addition to PmSTAT, WSSV is able to control other immune related pathways. Notable among the immune related pathways of signaling are Nuclear Factor-k-B and MAP kinase (Qiu et al., 2014). Genes those are expressed in different phases of infection were classified according to their functions as transcription factors, kinases, and ubiquitin E3 ligases. For successful replication of WSSV's genome, WSSV mainly uses DNA replication machinery of the host cell which ultimately stops the activity of the S-phage of the host cell though WSSV also has some DNA replication machinery of its own (Chen et al., 2002).

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The presence of a microorganism like WSSV changes the environment inside the crustacean cell. Host cell being affected by the presence of the virus becomes less conductive for deterioration of the environment of the cell where viral replication becomes harder. For keeping the environment under control to replicate successfully, WSSV counteracts to manage the demand for energy needed for anabolic reactions by inducing aerobic glycolysis, and essential nutrients like iron by interacting with host ferritin. On the other hand, hosts have also developed mechanisms to reduce viral replication inside the cell. They might signal for apoptosis inside the cell for self-destruction. Virus then controls such adverse situation by interacting with host metabolism, stress response system and apoptosis signaling (Fung et al., 2014). Unfolded protein responses (UPR) due to generation of misfolded proteins at Endoplasmic Reticulum (ER) are induced for the presence of WSSV in host cell and data suggest WSSV's capability to interact with the downstream effectors transcription factors that get activated by UPR aiding in the transcription of viral genes (Verbruggen et al., 2016). A very important aspect during WSSV infection is apoptosis which is regulated by caspases (initiator caspases and effector caspases) that are targets of WSSV. Autolytic processes activate initiator caspases and the effector caspases are there as zymogens that are activated through initiator caspases' cleavage. Repression of the action of an effector caspase (PmCaspase) of black tiger shrimp was observed by anti-apoptotic protein of WSSV (WSSV449) in Sf9 cells (Leu et al., 2010). WSSV134 and WSSV322 were found to bind another effector caspase PmCasp demonstrating anti-apoptotic activity in similar cell system like Sf9 (Lertwimol et al., 2014).

After WSSV's survival applying its anti-apoptotic activity, this virus which has a genome around 300 kb needs to get packaged inside the nucleocapsid. A small viral protein of 6.7 kDa, VP15, was found to be associated with this packaging. The information is still little on

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how the packaged virus gets released from the host cell.

1.2.4 Immunity

Immunological memory does not exist in crustaceans. Crustacean immune system is innate which includes humoral and cellular responses along with physical barriers (Lee and Söderhäll, 2002; Deris et al., 2020). Immune genes, antiviral compounds in tissues, RNA interference and presence of toll like receptors (TLR) are evidences of crustacean immunity suggested in studies though the exact role TLRs is not confirmed (Barton, 2007). Si-RNA (Small interfering RNA) injected crustacean demonstrated lower replication of WSSV. The challenged shrimps in which the anti-polysaccharide factors were upregulated demonstrated lower replication of virus. A number of immunity genes encoding proteins like oligo synthase and interferon were found in the hemocytes of shrimps which survived during an outbreak (He et al., 2005) indicating the presence of a WSSV-neutralizing substance in the survivor crustaceans. In an earlier study, Roux et al. (2002) informed about common defense mechanisms to different kinds of pathogens like β 1, 3 glucan binding protein and lipopolysaccharide which get upregulated after infection. In studies with challenge experiments, apoptosis was mentioned as a process to stop virus replication though virus has the capacity to fight employing immediate action called anti-apoptosis (Hameed et al., 2003). Two immunity genes Pen and Lyso might play a very important role in immunity by destroying cell walls of microbes through hydrolysis and which were found to upregulate in WSSV-infected shrimp to fight against WSSV (Deris et al., 2020). Deris et al. (2020) also mentioned that the gene expression levels of these immunity genes could very well illustrate immune conditions of the crustaceans affected by WSSV.

1.2.5 Immunologically the most important viral envelope protein, VP28

VP28 is immunologically one of the paramount crucial WSSV virion proteins which is involved in binding to the host receptor protein Rab7. VP28 sequences have been submitted in GenBank from many countries including China, Vietnam, Indonesia, USA, Japan, Korea, India, Thailand, Mexico, Brazil, The Netherlands, Iran, Bangladesh, Egypt, Philippines, Ecuador and Saudi Arabia. These countries have reported VP28 sequences from WSSV isolates of hosts including *Penaeus monodon*, *Penaeus japonicus*, *Penaeus setiferus*, *Penaeus indicus*, *Procambarus clarkia*, *Panulirus Homarus* (spiny lobster), *Macrobrachium rosenbergii*, *Litopenaeus vannamei*, *Scylla serrata*, *Scylla olivacea*, *Penaeus vannamei*, *Fenneropenaeus indicus*, *Marsupenaeus japonicus*, WSSV-Chimera, and *Penaeus vannamei*. VP28 has 204 amino acids. Major mutation points of VP28 includes 42nd and 114th amino acid positions and very rarely in 167th amino acid positions. Changes from consensus sequences are G from D at 42nd position, R from Q at 114th position and G from E at 167th amino acid position. In Bangladeshi isolates till 2014 to 2017, the very rare mutation at 167th amino acid position was observed in some samples. But none of the isolates had mutations at 114th position and all samples had G mutated from consensus D at 42nd position. On that basis, two groups are found, one having E at 167th amino acid position and another having G at the 167th amino acid position which was rarely found in the isolates of Egypt other than Bangladesh (Siddique et al., 2018).

1.2.6 Host receptor protein Rab7

Receptor is of substantial significance to the ecology of the virus. Appropriate receptors among species regulate the host variety of the virus, and the expression of receptors in cells of the hosts is one of the determinants that fixes the pathogenic consequence of infection (Verbruggen et al., 2016). Rab7 is a WSSV receptor protein found in chordates, annelids,

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cnidarians, amphibians, priapulids and arthropods including crustaceans and other groups. WSSV has a record of killing mainly penaeid shrimps and crabs which are cultured in ghers situated in coastal zones. Envelope protein VP28 of WSSV is known to be involved in systematic infection of shrimps through binding to the Rab7 protein of the host crustaceans (Sritunyalucksana et al., 2006). We think that envelope protein of WSSV may bind to Rab7 of some other organisms also which could act as vectors or asymptomatic carriers. Sritunyalucksana et al. (2006) are among the pioneers in reporting the involvement of penaeid shrimp's Rab7 protein in binding an envelope protein of WSSV known as VP28. The Rab7 identical sequences varied from 86.5 to 100% in all organisms while it varied from 94.6 to 100% in decapods that include shrimps and crabs. Protein–protein interactions are physical associations of higher specificity set between two or more protein molecules for biochemical actions directed by interactions which include hydrogen bonding, electrostatic forces and hydrophobic effect (Rivas and Fontanillo, 2010). It is publicized that structures of protein are more conserved than sequences of protein and DNA among homologs while visible levels of sequence similarity typically infer significant similarity in structure (Marti et al., 2000). Highly identical protein sequences show that this Rab7 is quite conservative in nature in the decapods. Although WSSV is known to bind to its receptor protein PmRab7 in *Penaeus monodon*, but there is also confusion regarding this. It is also mentioned that PmRab7 may not be responsible for the entry of the virus to the host. It may bind to the viral protein at a later stage (Zhou et al., 2009). It is also mentioned in the study of Zhou et al (2009) that there could be a multiprotein complex of different structural proteins of WSSV before binding to the host protein and at least five complexes of structural proteins had been mentioned for binding to the receptor (Xie and Yang, 2006,; Xie and Yang, 2015). The long conserved domains in Rab7 sequences with identical residues have the similar interaction interfaces. It was found in a study of Hameed et al (2002) that WSSV could not be infected to

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Artemia which is a crustacean at its developmental stage by immersion challenge which was confirmed by PCR negative detection mentioning Artemia might not be a carrier, though they suggested for more studies. On the other hand, it was possible to infect rotifer by artificial challenge. WSSV was also detected in rotifers from natural samples from shrimp ponds and the rotifer resting eggs by PCR detection mentioning rotifers as the vectors or carriers of WSSV to transmit to shrimp (Yan et al., 2004). WSSV was found in Pacific oyster *Crassostrea gigas* while its susceptibility to infection and role as a carrier was underdetermined, reported in another study (Vasquez-Buacard et al., 2010). Cornejo et al. (2017) observed prevalence of WSSV in zooplankton samples and maximum occurrence was found in taxa of copepods, brachyurous and bivalves. They also mentioned that 12 taxa can be high-risk vectors of WSSV and dispersion of WSSV though these planktons have not been estimated in years.

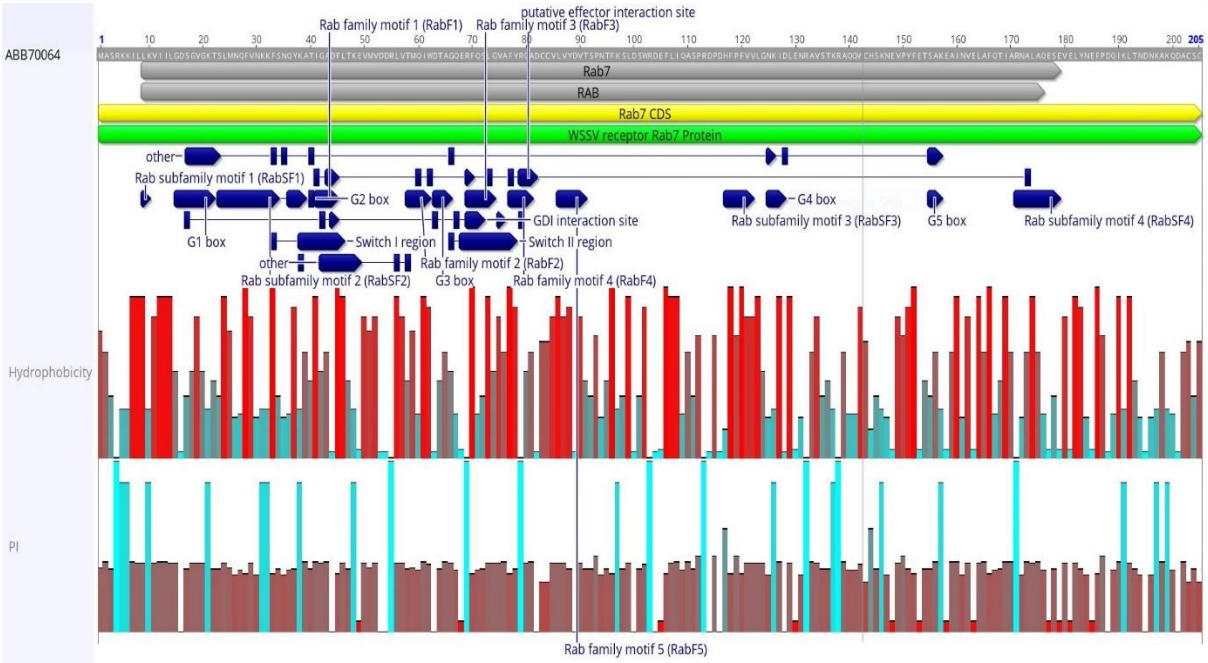

Figure 1.9 WSSV receptor rab7 protein isolated from *Penaeus monodon* [GenBank: ABB70064]. Molecular weight: 23.219 kDa, isoelecric point: 5.62, charge at pH 7: -2.21

The simulation and docking of *Pm*Rab7 (Verma et al., 2013) revealed Arg 69, Leu 73, Val

75, Arg 79 and Ala 198 as the active sites. Figure 1.9 has been drawn using Genious Prime Trial Version which shows the hydrophobicity and isoelectric point of the protein considering amino acid sequences from GenBank Accession no. ABB70064. This Figure also shows the different interaction sites of *Pm*Rab7.

1.2.7 Detection of WSSV

Numerous approaches including microscopic examination under light, in dark field, using phase contrast microscope and transmission electron microscope, by introducing bio-assay, using histopathological and molecular methods had been practiced for the diagnosis of WSSV (Lightner and Redman, 1998). Detection of WSSV was made possible by procedures based on immunology, conventional PCR (Lo et al., 1996; Otta et al., 2003), Multiplex PCR (Jeeva et al., 2014), quantitative PCR (Mendoza-Cano and Sanchez-Paz 2013), in situ hybridization (Chang et al. 1998) and also the loop-mediated isothermal technique (Chang et al., 1998). Out of the above mentioned methods, PCR is regarded as an exceptionally sensitive and delicate method to identify WSSV's presence (Lightner and Redman, 1998) and is widely used nowadays for prevalence study. Real-time PCR based methods (Sybr-green chemistry and Taqman probe based) are practiced for quantification of WSSV load in the samples where WSSV is detected. The amplification of particular DNA sequences using polymerase chain reaction (PCR) is accomplishing its pledge as the major diagnostic practice for the detection of such pathogens. These conventional PCR methods are based on primers which are designed targeting a particular piece of DNA sequence of the virus (Siddique et al., 2018). For the need of sensitive and high-tech diagnosis, researchers developed primers and probes after the first appearance of WSSV. One step diagnostic PCR and two step diagnostic PCR were used in the beginning for WSSV detection with dot and southern hybridization procedure. The main clinical symptom of the disease caused by WSSV is the occurrence of white spots in the exoskeleton and epidermis of shrimps which can be identified through

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physical observation (Siddique et al., 2018).

1.2.8 Quantification of WSSV

Quantification of load of WSSV in samples have been widely practiced using quantitative real-time PCR. Utilization of Sybr-green chemistry for quantification are common methods with particular mastermixes and primers, or probes. The real-time PCR is a modification of the conventional PCR technique to quantify DNA or RNA present in a sample using primers specific to target sequences. Amplification plot is generated from plotting fluorescence against the cycle (Siddique et al., 2018). If the specific sequence is present in ample numbers in a sample, the amplification will be observed in earlier cycles than the one where the sequence is scarce. ABI 7500 real-time PCR machine and BioRAD's CFX96 real-time PCR machines are widely used. Depending on the mastermix, real-time PCR run (denaturation, annealing and extension) properties are fixed. Recombinant plasmid based standard is used for the purpose of absolute quantification (Mendoza-Cano and Sánchez-Paz, 2013). Siddique et al. (2018) used recombinant plasmid which contained VP28 gene, inserted in TOPO TA vector. Gene expression profiling is done through DNA microarray analysis and real-time PCR assays. In DNA microarray assays, microarray chip was used for the analysis of transcriptomic response of crustaceans to WSSV infection (Shekhar et al., 2015). Profiling with real-time PCR is done after total RNA was extracted and cDNA was converted from total RNA. cDNA was synthesized for analyzing the relative expression levels of host genes (Shekhar et al., 2015) and viral genes (Jeena et al., 2012) through real-time PCR.

1.2.9 Determination of virulence properties by experimental infection

Shapiro-Ilan et al. (2005) tried to examine definitions of virulence and pathogenicity in invertebrate pathology. They defined pathogenicity as the capacity of causing disease in an organism while virulence is the power of an organism to cause disease. Pathogenicity was used as a qualitative and virulence was used as a quantitative term which quantifies the pathogenicity. Chen et al. (2000) observed mortality of *Scylla serrata* larvae which were experimentally infected by WSSV using immersion technique. They confirmed that the death was for the establishment of infection with WSSV by detecting the virus the samples using conventional PCR. Somboonna et al. (2010) by their single challenge and serial challenge study demonstrated that *Scylla olivacea* and *Scylla paramamosain* was susceptible to WSSV while S olivacea was more susceptible and also observed *Scylla serrata* to be more stable than both of the other two. They also mentioned that mortality after challenging *Scylla* spp. is species dependent through studying the quantity determination from the standard curve of C_T values and copy numbers of WSSV by expressing their results as mean copy numbers ± standard deviations (SDs) for the triplicate samples. Escobedo-Bonilla et al. (2007) experimentally infected shrimps through oral inoculation in glass aquaria which were equipped with water heater and continuous aeration. They detected virus at 18 hpi from the cell-free haemolymph of the shrimp samples and to detect early they used 1-step PCR. Gunasekaran et al. (2017) used the term ‘waterborne inoculation’ for challenging crabs with the filtrate of crab meat containing WSSV maintaining standard experimental conditions in culture tanks. For confirmation of the infection caused by WSSV, they used PCR-based molecular diagnosis by following the instruction of ‘ICAR-Central Institute of Brackishwater Aquaculture (CIBA) diagnosis kit’. Nayak et al. (2010) challenged *Penaeus monodon* larvae for checking upregulated genes like penaeidin and lysozyme in 2-litre glass containers for 3 days while regulation was assessed using Real-time PCR. Escobedo-Bonilla (2005) compared

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cumulative mortality and median lethal time (LT_{50}) by challenging *Litopenaeus vannamei* with WSSV strains to compare their virulence. Deris et al. (2020) challenged black tiger shrimp PL of different stages with *Vibrio parahaemolyticus* strain through immersion challenge test for checking the survival probability showing Kaplan-Meier curves and assessing expressions of immune related genes including Penaeidin and Lysozyme. Shekhar et al. (2015) infected black tiger shrimp with WSSV and observed gene expression by micro array and Real-time PCR. Jeena et al. (2012) infected shrimps by injecting WSSV and rearing in plastic crates with adequate aeration and studied expression of shrimp ubiquitin enzyme and WSSV ORF199. Marks et al. (2005) used virus in the tissue filtrate for experimentally infecting shrimps and determined the virulence of the isolates by observing cumulative mortality rates and quantitative fitness in serial passage by qPCR.

It was found reviewing most of the studies that virulence was determined by observing the severity of infection in challenged organisms tissue, disease onset time, median LT_{50} and LT_{100}. Previously there was no certain dose for testing organisms (Marks et al., 2005). Results varied due to testing on different organisms and with different doses. Virulence determination in laboratory conditions and the virulence in nature could vary as crustaceans might have to face unfavourable conditions in natural environment. Intramuscular injection, oral administration, immersion in suspension and feeding of WSSV-contaminated food had been practiced as the major inoculation procedures to challenge shrimps and crabs to observe the virulence. The virulence may also vary for different methods implied for treating organisms. However, these techniques practiced on crustaceans are relatively good enough for illustrating relative virulence of variants of WSSV in laboratory. In the crustaceans challenged with WSSV through different methods, infections of WSSV were found in ovaries, testes, eyes, pereiopods, nervous tissues, midgut, hindgut, pleopods, muscle tissues,

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heart, hepatopancreas, lymphoid organ, connective tissues, hematopoietic tissues, cuticular epithelium, stomach, gills and haemolymph (Escobedo-Bonilla et al., 2007). Major tissues targeted by WSSV for its replication are gills, stomach and cuticular epithelium, antennal gland and hematopoietic tissue which get lysed and degenerative in the later stage of infection (Chang et al., 1996). WSSV spreads to reach the hemal sinuses crossing the basal membrane after replicating primarily in the mentioned tissues though primary replication is influenced by the procedure of inoculation. To cause the new wave of infections, WSSV spreads in the crustacean organs circulating through haemolymph (Escobedo-Bonilla, 2007). However, WSSV could be a sole pathogen in the challenge experiments held in laboratories, but there could be differences in virulence in natural conditions for different biotic and abiotic factors along with the chances of co-infections through different other pathogenic viruses and bacteria (Umesha et al., 2006).

1.3 Hypothesis of the study

WSSV is a highly prevalent serious crustacean pathogen reported regularly by the traditional farmers of shrimps and crabs of the coastal regions in Bangladesh. Assessment of the prevalence of WSSV in shrimps and crabs and any impact of physico-chemical parameters on the prevalence of WSSV in the ghers of both the coastal regions should be conducted regularly. To find out the relative virulence properties of WSSV phylotypes circulating in Bangladesh through experimental challenge studies was of utmost importance. This study also targeted to explore the gene expression profiling of Penaeidin and Lysozyme in the shrimp PL challenged with VP28 based phylotypes of WSSV to justify the virulence properties. This study was based on the hypothesis that WSSV's prevalence study in different geographic locations along with the knowledge of the virulence of the circulating WSSV will help in proper management of commercially important crustacean aquaculture and complete

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eradication of the disease through sustainable aquaculture practices.

1.4 Objectives

This study had the overall objective to monitor the prevalence of WSSV in selected improved traditional ghers of two coastal districts of Bangladesh where livelihoods of many people depend on crustacean aquaculture, and to understand the virulence of circulating phylotypes of WSSV across Bangladesh through experimental challenge studies. To fulfill the targets, the study had the following specific objectives.

(1) To detect and quantify WSSV through conventional PCR and real-time PCR based methods for studying prevalence and to monitor physico-chemical parameters of selected shrimp ghers

(2) To assess the relative virulence of VP28 based prevalent phylotypes of WSSV in crustaceans of Bangladesh through experimental infection assay.

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Chapter 02:
Materials and Methods

2.0 Materials and methods

2.1 Research plan

In this study, we had two working objectives, and workflows were prepared for both. For prevalence study, WSSV was detected using conventional PCR, and the real-time PCR was used for WSSV's load determination in the target crustaceans (Figure 2.1). For relative virulence study, experiment was designed with treatment groups and control groups (Figure 2.2).

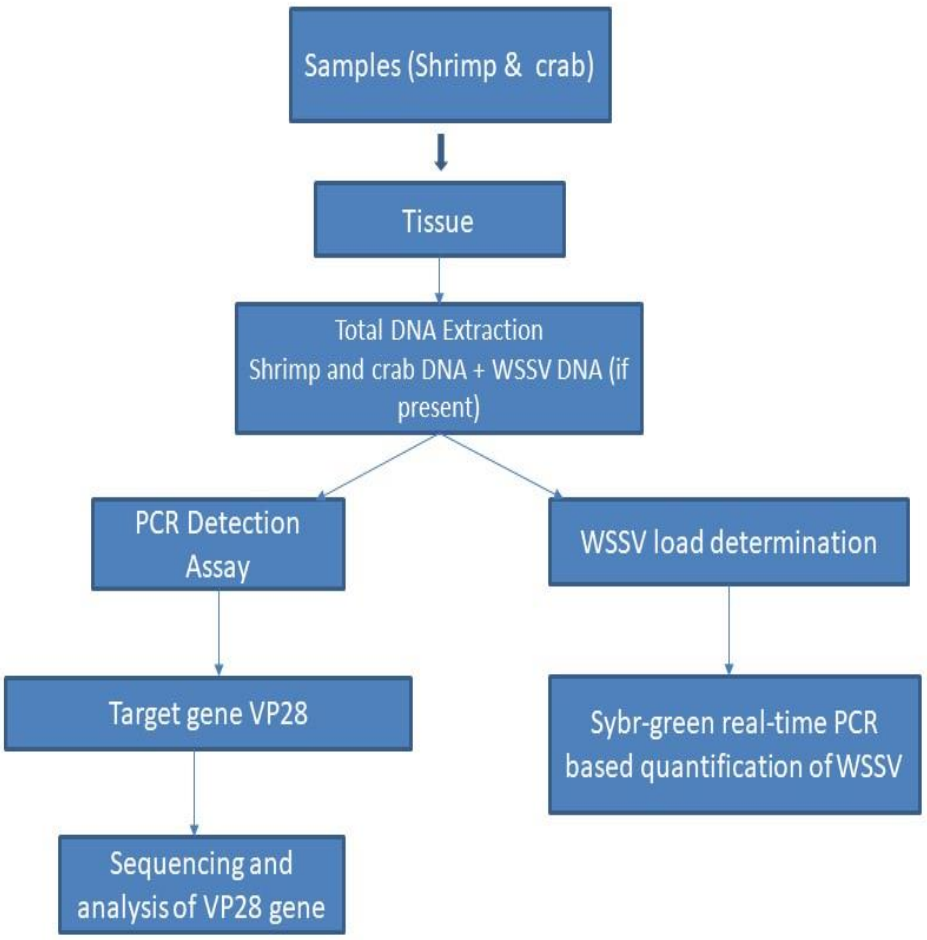

Figure 2.1 Workflow for prevalence and load determination

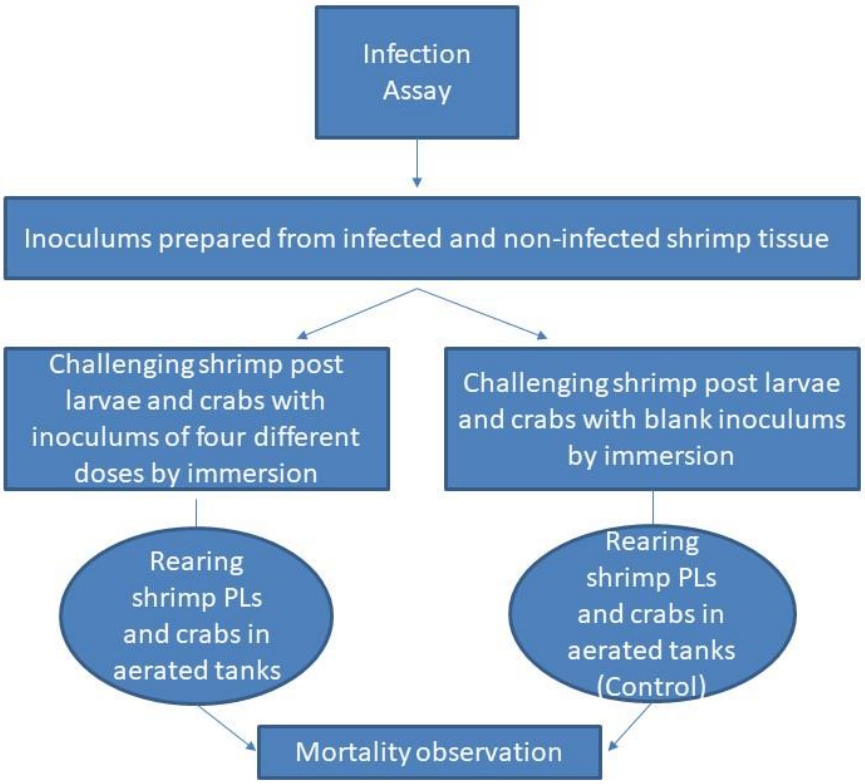

Figure 2.2 Workflow for the virulence determination through mortality observation in infection assay

2.2 Experimental crustaceans

We studied the prevalence of penaeid shrimps and mud crabs that are available in the different farming regions of Bangladesh.

2.2.1 Penaeid shrimps

In this study, *Penaeus monodon* was selected for the prevalence study because it is commercially the most important crustacean in Bangladesh. *Penaeus monodon* also known as black tiger shrimp is an arthropod having characterized by jointed appendages and exoskeleton which is molted periodically. It belongs to the subphylum Crustacea and class Malacostraca.

2.2.2 Mud crabs

Mud crabs are crustaceans which fall into the class Malacostraca as well like penaeid shrimps.

Bangladeshi mud crabs belong to the genus *Scylla* and the most common mud crabs are *Scylla olivacea* and *Scylla serrata*. Rouf et al. (2016) confirmed the mud crab species *Scylla olivacea* caught from these coastal regions of Bangladesh by genetic analysis of the partial sequences of one mitochondrial gene, 12S rRNA, morphological characteristics and morphometric ratios.

2.3 Sampling and measurement of physicochemical parameters

2.3.1 Sampling areas

Satkhira is a coastal district which is located in the southernmost region of Bangladesh. 32% of this district's area (aproximately 66800 ha out of 211000 ha) entails shrimp farms and contribute 34% (23400 metric ton out of 69000) of the production of shrimps entirely in Bangladesh (FRSS, 2017) (Figure 2.3-2.4A). Location of another coastal district, Cox's Bazar, is in the south-east region of Bangladesh. Shrimp and crab aquaculture practice takes place approximately in 41594 ha area of Cox's Bazar (Figure 2.3-2.4B). Part of the Bay of Bengal near to Cox's Bazar is known for having mother shrimps. For that reason, most of the shrimp hatcheries are located in this south-east coastal region.

Shrimp and crab samples were collected from Sadar Upazilla of Cox's Bazar District and 5 upzilla, Satkhira Sadar (SS), Debhata (D), Asassuni (A), Kaliganj (K) and Shyamnagar (S) of Satkhira District. In this current study, crustacean samples were obtained from 20 ghers during the period of 2017-2019 when farmers informed presence of WSD in the region. Satkhira is contributing a lot in the aquaculture sector of Bangladesh, and presence of WSSV has been reported more in this district compared to Cox's Bazar district in the recent years. Therefore, we selected five Upazilla from Satkhira, and three (03) ghers were selected from each of these Upazilla. On the other hand, we selected five (05) ghers from the Cox's Bazar sadar Upazilla. However, the total number of shrimps and crabs in the given facility was unknown for the farmers as we collected samples from improved traditional ghers.

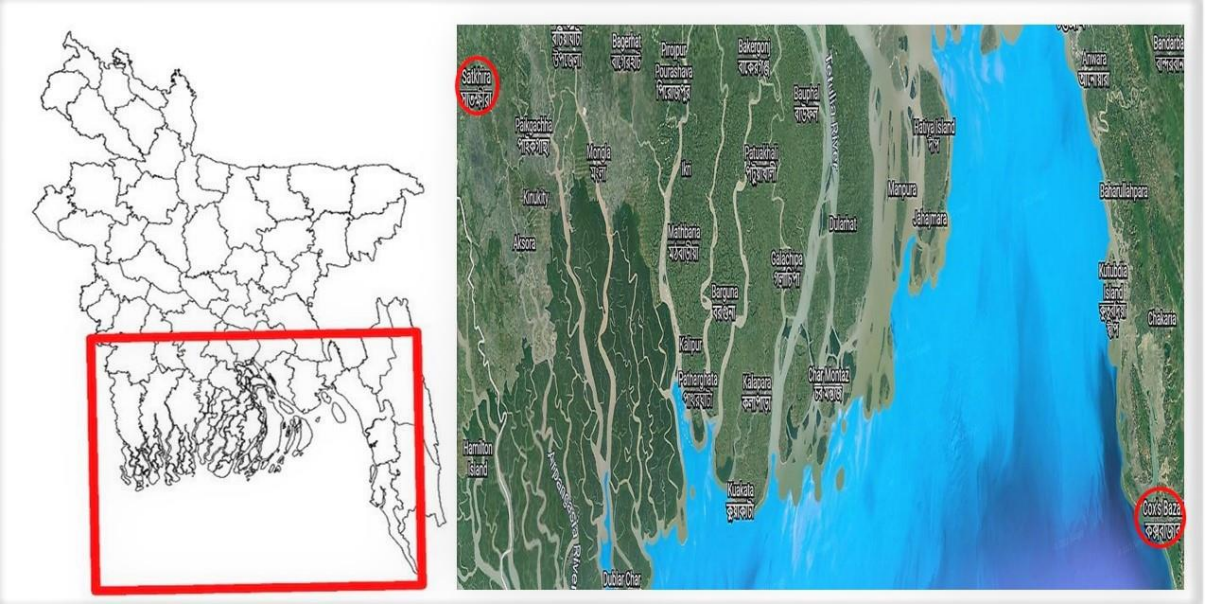

Figure 2.3. South-east and south-west coastal regions of Bangladesh (marked using red colour) where shrimp ghers are located

(A) (B)

Figure 2.4 (A) Shrimp gher at Satkhira's Kaliganj Upazilla, (B) Google map image of sampling ghers at Cox's Bazar

2.3.2. Measurement of physico-chemical parameters

Physico-chemical parameters of the shrimp ghers of sampling regions were monitored in the morning during the study period. Temperature, *in situ*, was measured using centigrade thermometer. Salinity was also measured *in situ* using refractometer. Dissolved oxygen of the shrimp gher water were estimated according to APHA (1998). Digital pH meter from Hanna instruments was used for the measurement of pH *in situ*.

2.3.3 Collection of samples

After growers complain concerning mortality of the crustaceans in the improved traditional farming ghers (ponds) of both the selected regions, 360 shrimps and 120 crab samples were collected during monsoon in 2017 to 2019 for the detection of WSSV using conventional PCR. Our sampling was also continued in post-monsoon throughout the three-year study period with the observation of same number of samples when crustaceans' death was not reported by the respective farmers of the ghers in the research areas.

Figure 2.5 shows the on-field samples during sampling and before transporting to the laboratory.

2.3.4 Transportation of samples

The samples were collected from the culture area when farmers reported that shrimps were getting diseased in their ghers. Preliminarily, WSSV was spotted by visual observation of white spots in the carapace of shrimps. After the primary visual observation, samples were instantly brought to the laboratory for additional analysis maintaining cold chain (below 4ºC) efficiently, and were stored at -20 ºC until further diagnosis.

(A)

(B)

(C)

(D)

Figure 2.5 A. Shrimps found dead in cage, (B) dead crab in a shrimp gher, (C) shrimp samples collected containing white spots, and (D) collection of a dead mud crab from a study gher

2.4 DNA extraction

2.4.1 Automated DNA extraction

After preliminary examination considering signs of infection, DNA from tissue was extracted by using automated DNA extraction system (MaxWell 16® Tissue DNA Purification kit; AS 1030, Promega, USA), according to manufacturer's instruction (Siddique et al., 2018). Prior to the extraction of DNA from WSSV-treated shrimp PL, PL tissue were collected in sterile 1.5 ml microfuge tubes and smashed into fine particles using glass rod.

2.4.2 Measurement of DNA concentration

NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) was employed to evaluate the purity and concentration of DNA extracted from shrimp and crab tissue samples (Hossain et al., 2015). The measurement unit of the product was ng/µl.

2.5 Conventional PCR

The obtained DNA experienced a conventional PCR test for the amplification of the VP28 gene making use of GoTaq 2 X Hot Start Colorless Master Mix (Promega, USA) with forward and reverse primers (Rout et al., 2007, Siddique et al., 2018). The primer sequences for the amplification of VP28 gene were VP28F 5´-GCGCGCGGATCCAATCATGGATCTTTCTTTCAC-3´ and VP28R 5´-GCGCGCGAATTCTTACTCGGTCTCAGTGCC- 3´. The conventional PCR reactions involved denaturation at 95 ˚C for 50 minutes, annealing at 55 ˚C for 30 seconds, extension at 72 ˚C for 45 seconds, and continued for 30 cycles with a final extension of 5 minutes at 72 ˚C. Amplicons of PCR were visualized on 1.0% agarose gel in TAE buffer stained with

ethidium bromide. After agarose gel electrophoresis, the bands were photographed under UV light (Figure 2.6A).

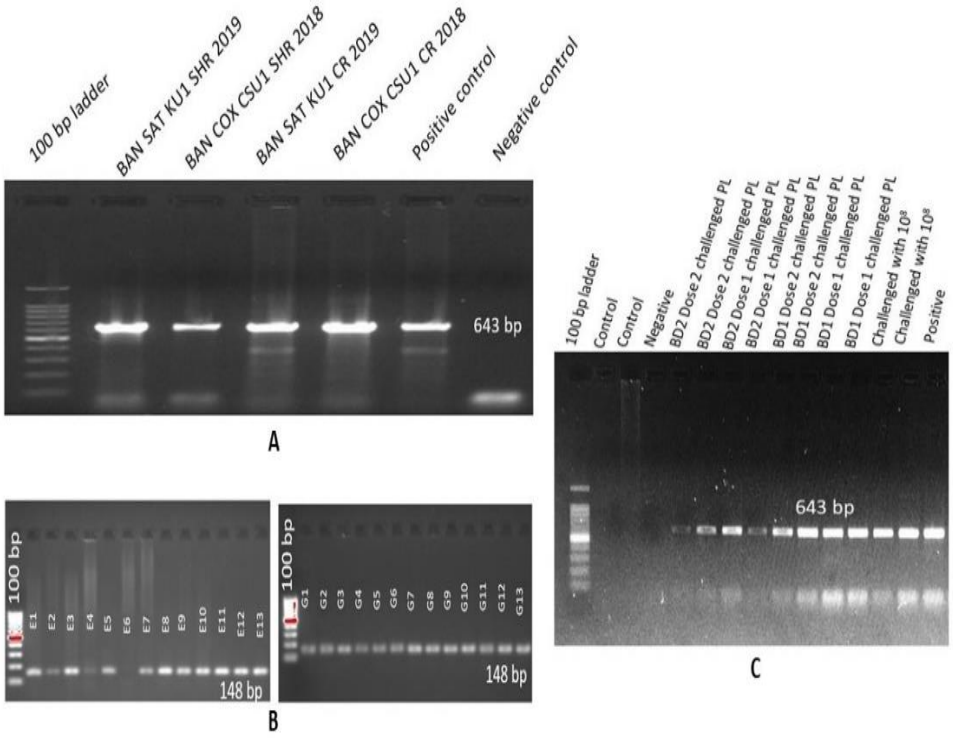

Figure 2.6 A. Lane 2: positive shrimp sample from Satkhira, lane 3: positive shrimp sample from Cox's Bazar, lane 4: positive *Scylla olivacea* sample from Satkhira, lane 5: positive *Scylla olivacea* sample from Cox's Bazar, lane 6: positive control, lane 7: (Negative control) sample in which WSSV was not detected; B. 148 bp products represent detection confirmation in real-time PCR products from both challenged groups (E and G represent samples of BD1 and BD2, respectively); C. Lane 2-3: Samples from control group showing no band, lane 4: No template control, lane 5-14: Challenged PL positive in conventional PCR, and lane 15: positive control (Lane 1s in A, B and C represent 100 bp ladder)

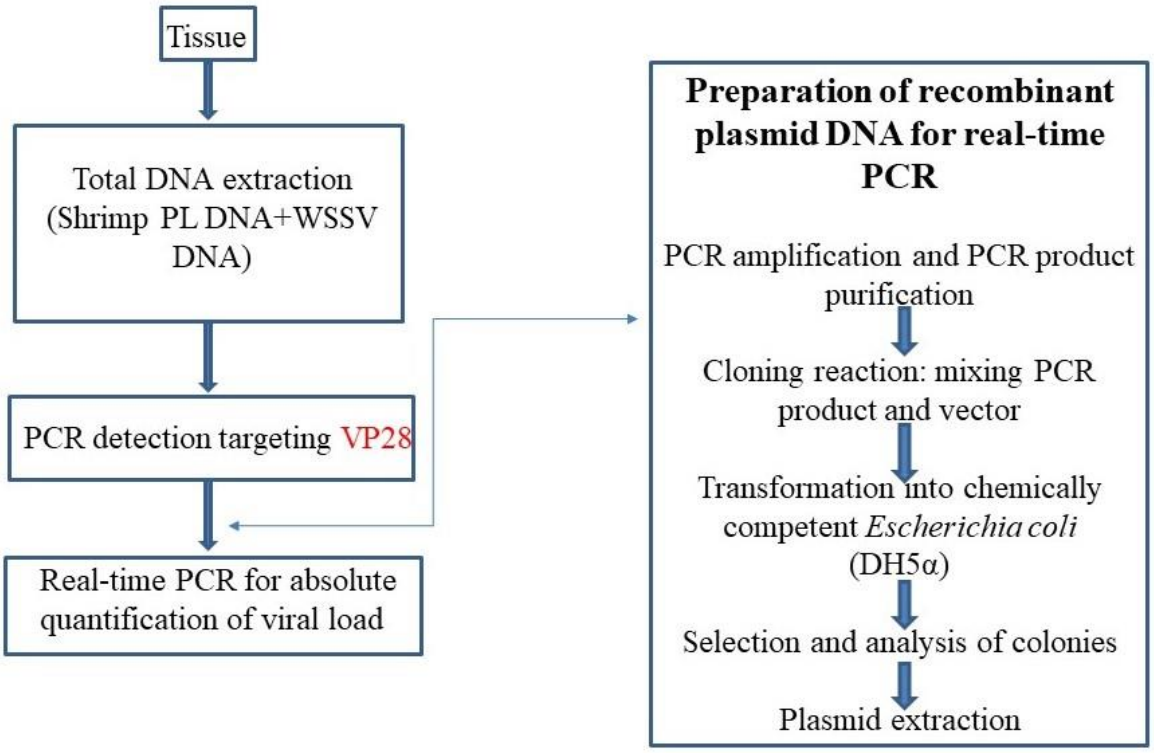

Figure 2.7 Preparation of recombinant plasmid DNA for real-time PCR to quantify viral loads

2.6 Quantitative real-time PCR (qPCR) assay

2.6.1 Preparation of recombinant plasmid DNA based standard

Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) was used to purify the recombinant plasmid which contained the VP28 gene (643 bp PCR product, complete CDS of VP28 gene inserted into TOPO TA Vector). The recombinant version of the plasmid was created in a ligase independent manner. The protocol of the preparation of the recombinant plasmid DNA has been postulated in Figure 2.7. The amplified 643 bp PCR product was purified prior to cloning reaction and then transformed into chemically competent *Escherichia coli* DH5α. After analysis and the selection of the colonies, plasmid was extracted. NanoDropTM spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) was applied to estimate the concentration of recombinant plasmid. The

measurement unit of the PCR product was ng/µl. In a linear logarithmic scale of 1.0 X 10^9 to 10^2 copies per reaction, purified recombinant plasmid was serially diluted to prepare the standard. Prior to the serial dilution, mass of the recombinant plasmid DNA was determined using the formula, m = [n] [(1/6.023×10^{23})] [660] g, where n is the size (bp) of the plasmid, m represents the mass of plasmid, 6.023×10^{23} molecule/mole is the Avogadro's number and molecular weight (average) of the ds DNA is 660 g/mole. In this study, size of the vector and insert was 3956 bp and 643 bp, respectively. So, the mass of the recombinant plasmid was determined as 5.040504 × 10^{-9} ng. For the preparation of the serial dilution of the plasmid, the formula used was the C1V1=C2V2, where C1 represents the initial DNA concentration, C2 final DNA concentration, V1 volume to be taken from stock, and V2 final volume.

2.6.2 Reaction mixture and cycling parameters

All qPCR reactions had been run at a final volume of 25 µL in the Applied Biosystems® 7500 Real-Time PCR system (Foster City, CA, USA) using 2 X SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 100 nM of each of the forward and reverse primers and variable quantity of individual template DNA. The real-time qPCR was run with primer pair WSSV-q28F 5′-TGTGACCAAGACCATCGAAA-3′ and WSSV-q28R 5′-CTTGATTTTGCCCAAGGTGT-3′ checking on the previously established techniques with little modification (recombinant plasmid based standard instead of purified PCR product based standard) (Siddique et al., 2018, Mendoza-Cano and Sánchez-Paz, 2013). The reaction mix was gently centrifuged creating no bubbles when the template was added. Experimental mix was shielded from light in the refrigerator until further use as unnecessary exposure to light could have an effect on the fluorescent dye.

Parameters of thermal cycling had been set for an initial denaturation phase at 95 °C for 10 minutes followed by 40 cycles at 95 °C for 15 seconds for DNA denaturation with subsequent annealing and extension at 53 °C for 30 seconds. Assessments of melt-curve were performed as well to distinguish specific amplicon from primer dimer or non-specific product amplification. Additionally, the qPCR products were also taken for electrophoresis in agarose gel to refute the existence of any spurious amplicon by observing specific band size. The current experiment was performed with replicates to count the copy numbers of virus.

2.6.3 Viral load quantification

WSSV load per gram of tissue sample was determined using the following procedure of calculation:

Viral load per gram of tissue = [viral load per reaction X (Final Elution volume/volume of template DNA per reaction) X dilution factor] ± Standard Deviation (SD).

To evaluate the ability of replication of the standard curve, standard reactions had been generated thrice separately with replications of each reaction. The quantitative real-time PCR records were assessed by making use of 7500 software, version 2.0.6 (Applied Biosystems, Foster City, CA, USA). The observed data were brought under analysis by the use of statistical program Microsoft Excel 2020 and stated as mean ± SD. Standard deviation of the observed viral load for each reaction was considered in the course of the viral load calculation (Siddique et al., 2018). For experimenting the relative virulence, the viral copy numbers in the WSSV treated shrimp PL were quantified per reaction with same amount of initial concentration of DNA. The VP28 gene amplified through real-time PCR produced a 148 bp product (Figure 2.6B).

2.7 Sequencing of VP28 protein, phylogenetic and mutation analyses

Wizard® SV Gel and PCR Clean-Up System (Promega, USA) were used to purify the conventional PCR amplicons, and the seven (shrimp = 5; crab = 2) cleaned PCR products were exposed to an automated dideoxy cycle sequencing reaction with BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems®, USA) according to the manufacturer's instruction (Hossain et al., 2015). Sequence cleaner (https://github.com/metageni/Sequence-Cleaner) with set parameters of minimum length (m = 3,822), percentage N (mn = 0), keep_all_duplicates, and remove_ambiguous was applied to remove all ambiguous, and low-quality sequences (Rahman et al., 2020). The raw data of the sequences were put together for assembling through SeqMan version 7.0 (DNASTAR, Inc., Madison, WI, USA) and the sequences assembled were contrasted with other several entries from NCBI GenBank database (Benson et al., 2016) with BLAST (Altschul et al., 1990) search to unveil the identification, and for corresponding with VP28 envelope protein gene of WSSV. Applying 'Molecular Evolutionary Genetics Analysis' (MEGA) version 7.0 for larger datasets (Kumar et al., 2016), the sequences of VP28 gene from the isolates were considered for alignment with each other, and with appropriate reference sequences from our earlier study (n = 17) and NCBI GenBank database (n =10), with >90% taxonomic identity. The tree generated was a maximum-likelihood with the Tamura-Nei evolutionary model (Kumar et al., 2016, Saha et al., 2021). Nodal confidence in the resultant phylogenetic associations was analysed using the bootstrap test (1000 replicates) (Hoque et al., 2020). VP28 sequences of WSSV's different isolates of shrimp and crab along with 143 reference sequences of the VP28 retrieved from GenBank were considered for a multiple alignment through MAFFT (Katoh et al., 2009), and few adjustments were done by manual editing. The repeat units of each one of the isolates were annotated by applying Geneious Prime (Trial Version), and considered for alignment in

contrast to a reference sequence for amino-acid (aa) variability score counting (Rahman et al., 2021b, Rahman et al., 2021a).

2.8 Experimental infection

Meghna Shrimp Hatchery is situated in Ukhia, Cox's Bazar where mother shrimps are brought from the Bay of Bengal. The Manager of the Hatchery agreed to give *P. monodon* post larvae (PL) with a length of 1-2 cm. In this hatchery, the WSSV-free wild-caught mother shrimps' eggs are used for the purpose of hatching these penaeid shrimp PL. WSSV-negativity of the PL were confirmed by conventional PCR after transporting those to the laboratory before the challenge studies were performed. Tissue from highly infected crustacean was used to prepare WSSV inoculum. Tissue taken from the below part of the carapace of infected shrimp was minced and homogenized in the sterile sea water. The supernatant was stored after centrifugation at 8515 X g for five minutes which was filtered by the use of 0.45 µm membrane soon after. The stock was diluted to formulate infective dose containing 10^8 copies of the virus per mL. From a WSSV-negative shrimp sample, blank inocula were prepared. We performed the challenge experiments which was previously established as 'immersion technique' (Chen et al., 2000) and as 'waterborne inoculation' (Gunasekaran et al., 2018). The trial groups (n = 180, in each jar) were treated with WSSV irrespective of any phylotype by immersion technique in aerated glass jars with WSSV solutions in three treatments. Using the blank inoculum (prepared from WSSV-negative tissue), aerated jars were set for the control group (n = 180, in each jar). Commercially available artificial diet was given to the PL once per day at the rate of 10% body weight. PL mortalities were observed on an hourly basis, and the confirmation of the presence of WSSV was done by conventional PCR (Figure 2.6C).

Experiments for relative virulence determination were conducted in separate trials which included three treatments with shrimp PL (n=360, in each jar) challenged with inocula having equal number of copies of WSSV from both phylogroups (BD1 and BD2). The negative control groups with three treatments (n = 360, in each jar) were supplied with inoculum produced from WSSV-negative blended tissue. Hence, inocula were formulated by the mentioned steps from crustacean tissue containing equal copy numbers of WSSV (4.27 X 10^9) from both the groups and were added in the small aquariums for challenging. Infective doses contained filtrates from tissue of both groups with 0.423 X 10^9 and 0.423 X 10^7 copies of virus per mL in the sterile sea water. The water of the experimental tanks had a temperature that ranged between 28-29 ˚C and at the same time the salinity and dissolved oxygen were maintained 18 ppt and 5-6 ppm, respectively during the period of the trials. Mortality rates with time were assessed by counting the number of deceased shrimp PL in every six hrs. DNA was extracted from the experimented PL of WSSV-treated and the negative control group, and examined for viral load assessment by quantitative real-time PCR. Comparison was made through observation of the C_T values and the copy number of WSSV of different samples from the infection assay.

Mud crabs were brought under a pilot infection trial conducted through ingestion method on 45 mud crabs which were 6 months of age. Crabs for the challenge study were collected from a WSSV-free crab farm in the district of Satkhira which were diagnosed further by conventional PCR before the experiment. Crabs were treated with highly infected tissue on Day 1 and trash fish from Day 2 while crabs that were used as the negative control group were supplied with beef liver from the Day 1 (Gunasekaran et al., 2018). The treated groups of the crabs were given crustacean tissue containing both groups of WSSV (1.0 X 10^9) for ingestion. Experimental conditions of the challenge study with crabs were kept similar with

the virulence determination experiment of the shrimp PL except the salinity which was maintained at a higher level (28-30 ppt) for crabs. Agarose Gel Electrophoresis after PCR assay was conducted to confirm detection of WSSV in the experimentally infected crabs (Figure 2.8).

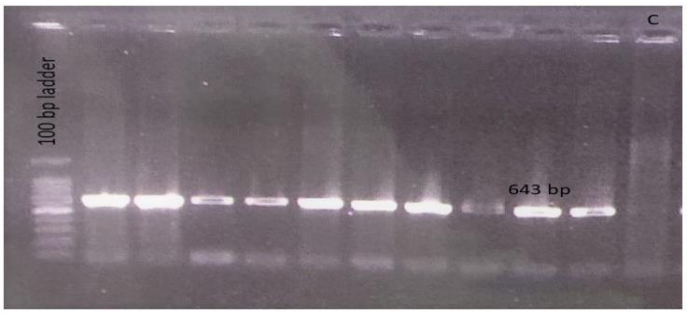

Figure 2.8 Lane 1: 100 bp ladder, lane 2-11: infected crabs after exposure to WSSV, and lane 12: crab from control group (no band in the control confirmed samples of other lanes showing bands were positive)

2.9 Gene expression

2.9.1 RNA extraction

QIAgen's QIAamp Viral RNA mini kits were used according to the protocol of the manufacturer for the purpose of extraction of RNA from the challenged PL and PL of the control group. Assessment of the purity and concentration of the extracted RNA was done using Nanodrop-2000 spectrophotometer.

2.9.2 Preparation of cDNA

Reverse transcription of the extracted RNA for synthesis of cDNA was required for the analysis of gene expression in Real-time PCR for studying any impact on the virulence assessment. New England Biolab's cDNA kit (PhotoScript II First Strand cDNA Synthesis Kit) was used for the purpose of reverse transcription.

2.9.3 Gene expression analysis using real-time PCR

Penaeidin and Lysozyme, two immunity genes of shrimps, were considered for the analysis of relative gene expression using real-time PCR. Such quantitative PCR was set for running with one cycle of initial denaturation at 95 °C temperature for 60 seconds and 45 cycles of denaturation at 95 °C for 15 seconds and extension at 60 °C for 30 seconds making use of the New England Biolab's Luna Universal qPCR master mix that was applied according to the protocol of manufacturer (Table 2 presents the list of primers). Comparative delta C_T method was used for the analysis of average-fold difference in gene expression (Jeena et al., 2012). For normalization, housekeeping gene *Beta-Actin* was applied which presented the C_T values as internal (endogenous) control. Any amplification in the samples of control groups that were inoculated with WSSV-negative tissue was considered for the analysis of gene expression. Calculation of Delta C_T was done by deducting C_T value of housekeeping gene (endogenous control) from the target gene, and in the end, mean delta C_T was estimated from the standardised value of delta C_T. Delta delta C_T was computed with reference to control by deducting mean delta C_T of control from mean delta C_T of the target gene. Changes in average-fold gene expression in the experimentally infected shrimp PL was calculated to $2^{-\text{delta delta } C_T}$ values.

2.10 Observation of binding affinity of VP28 and its receptor protein Rab7

The binding affinity of WSSV envelope protein VP28 of both groups from Bangladesh and receptor protein Rab7 were observed using the web server PROGIDY (Vangone and Bonvin, 2015; Xue et al., 2016)). VP28 sequences with glycine at the 167th amino acid position and VP28 sequences with glutamic acid at the 167th aa position were considered for the purpose of docking with Rab7 of *P. monodon* by HADDOCK (High Ambiguity Driven protein-

protein DOCKing) (van Zundert et al., 2016). Active residues (Verma et al., 2013) were produced from both molecules for the purpose of protein-protein docking. Before docking,

Table 2. Primers used in the current study.

Name of gene	Primer Sequence (5´-3´)	Reference
Penaeidin	F: TGGTCTGCCTGGTCTTCCT R: AAGCACGAGCTTGTAAGGG	(Deris et al., 2020)
Lysozyme	F: TGGTGTGGCAGCGATTATG R: GATCGAGGTCGCGATTCTTAC	(Deris et al., 2020)
Beta-actin	F: CCCTGTTCCAGCCCTCATT R: GGATGTCCACGTCGCACTT	(Shekhar et al., 2015)
VP28	F: GCGCGCGGATCCAATCATGGATCTTTCTTTCAC R: GCGCGCGAATTCTTACTCGGTCTCAGTGCC	(Rout et al., 2007)
qVP28	F: TGTGACCAAGACCATCGAAA R: CTTGATTTTGCCCAAGGTGT	(Siddique et al., 2018)

the Rab7 sequences were retrieved from the database of NCBI and the homology modelling of Rab7 was accomplished by means of using SWISS-MODEL and authenticated further by generating Ramachandran plots (Biasini et al., 2014). PROGIDY utilizes interactors from both proteins for serving the purpose of generating binding affinity (ΔG) and dissociation constant (K_d) values. Furthermore, web server DynaMut was used for observing any impact of such mutation to the stability of the protein structure of VP28 (Rodrigues et al., 2018).

2.11 Statistical analysis

Temperature, salinity, dissolved oxygen and pH were compared to the presence or absence of WSSV in the ghers. Non-parametric independent sample tests were conducted for salinity, temperature and dissolved oxygen, and parametric independent sample test was done for pH. Acceptance or rejection of null hypothesis was done of the distribution of physicochemical

parameters across categories of WSSV. When no significant interactions exist between time and isolate, the probit model holds the form: Probit (x) = α + β time + γ isolate, where α denotes the intercept, β is the rate of change in probability per unit change of time (for a constant isolate), and γ is the rate of probability. Eta test statistic was applied to examine the associations between WSSV (nominal variable) and the physicochemical parameters (scale variables). Furthermore, the mean prevalence of WSSV between areas was determined by the use of SPSS (SPSS, Version 23.0, IBM Corp., NY USA) (Hoque et al., 2019). At 5% level of significance using t-test, final values for average-fold differences in gene expression were analyzed.

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Chapter 03:
Results

3.0 Results

3.1 Prevalence of WSSV in the study ghers

There was a significant difference ($p < 0.05$, Kruskal Wallis test) in WSSV's overall prevalence during the study period of 2017 to 2019 between the study areas (Cox's Bazar and Satkhira) in shrimps and crabs. The average prevalence of WSSV in these crustaceans were 20.93% and 12.73% in the ghers of Cox's Bazar, and 16.73% and 9.53% in the ghers of Satkhira, respectively (Figure 3.1A). Year-wise comparison of the occurrence of WSSV in shrimps showed higher prevalence in the shrimp ghers of both the study areas (Cox's Bazar (23.11%) and Satkhira (18.96%)) in 2017, infection rates of WSSV in shrimps gradually declined in 2018 and 2019 subsequently (Figure 3.1A). On the contrary, WSSV's prevalence in crabs was observed to be at peak in the ghers of Cox's Bazar (14.16%) in 2019 and Satkhira (10.54%) in 2018 (Figure 3.1A).

3.2 Physicochemical parameters of the study ghers

In all the three years, mean values of salinity, dissolved oxygen, temperature and pH ranged from 14-16 ppt, 3.6 to 3.8 ppm, 33.9 to 34˚C and 7.8-8.4, respectively in the WSSV-positive ghers, and 13-21 ppt, 2.61 to 6.06 ppm, 28.7 to 34˚C and 8.2 to 9.1 in WSSV-negative ghers (Table 2, Table 3). Higher temperature in water in the WSSV-positive ghers in comparison to WSSV-negative ghers across the studied ghers of both regions along with higher salinity in the WSSV-negative ghers when compared to WSSV-positive ghers were two important findings of this study after observing the values of all the physico-chemical parameters (Figure 3.1B).

Table 3. Physicochemical parameters in the shrimp and crab ghers.

Gher ID	Salinity (ppt)	Dissolved oxygen (ppm)	Temperature (˚C)	pH	Shrimp samples selected	Crab samples selected
Cox1	14	3.8	33.9	8.4	18	6
Cox2	21	3.75	33.7	8	18	6
Cox3	21	3.65	33.7	7.8	18	6
Cox4	20	3.90	33	8.1	18	6
Cox5	20	3.70	32.9	8.2	18	6
SS1	14	3.8	34	8.5	18	6
SS2	15	3.6	33.7	8.6	18	6
SS3	15	3.7	34	7.8	18	6
D1	19	3.8	33.8	7.8	18	6
D2	17.5	3.7	33.5	8	18	6
D3	20	3.6	33	8.6	18	6
A1	16.5	3.5	33.3	7.7	18	6
A2	16	3.8	33.8	7.8	18	6
A3	15	3.6	34	8	18	6
K1	14	3.8	34.6	8.6	18	6
K2	15	3.6	34.7	7.8	18	6
K3	16	3.6	33.9	7.9	18	6
S1	13	3.6	33.9	7.9	18	6
S2	16	3.8	34	8.1	18	6
S3	16	3.7	34	8	18	6

Gher prefix started with C represents the ghers from Cox's Bazar while others represent the ghers of Satkhira.

3.3 Detection of WSSV in *Scylla olivacea*

WSSV was detected in the mud crabs (*Scylla olivacea*) collected from both the regions, Cox's Bazar and Satkhira. Visualization of 643 bp PCR product after Agarose Gel Electrophoresis validated the existence of WSSV in crabs (Figure 2.6A).

Table 4. Physicochemical parameters in the shrimp ponds when no WSSV was reported in the areas.

Gher ID	Salinity (ppt)	Dissolved Oxygen (ppm)	Temperature (˚C)	pH	Shrimp samples collected per year	Crab samples collected per year	WSSV-positive shrimp (%)	WSSV-positive crab (%)
SS1	18	4.79	29.2	8.5	6	2	0	0
SS2	16	3.12	29.2	8.6	6	2	0	0
SS3	19	2.61	29	8.5	6	2	0	0
D1	20	5.57	29.9	8.5	6	2	0	0
D2	17	6.06	30	8.5	6	2	0	0
D3	18	5.10	29.7	9.1	6	2	0	0
A1	19	4.2	30	8.67	6	2	0	0
A2	19	3.05	30.2	8.45	6	2	0	0
A3	19	2.46	30.3	8.5	6	2	0	0
K1	15	5.62	32.2	8.2	6	2	0	0
K2	20	7.67	33.2	8.5	6	2	0	0
K3	19	6.05	33.7	8.8	6	2	0	0
S1	17	5.85	29.6	8.5	6	2	0	0
S2	16	5.88	28.7	8.6	6	2	0	0
S3	20	5.37	28.9	8.4	6	2	0	0
Cox1	19	4.9	30.0	8.7	6	2	0	0
Cox2	18	5.20	30.1	8.6	6	2	0	0
Cox3	19	5.00	30.0	8.6	6	2	0	0
Cox4	19	4.5	30.3	8.5	6	2	0	0
Cox5	17	4.5	30	8.6	6	2	0	0

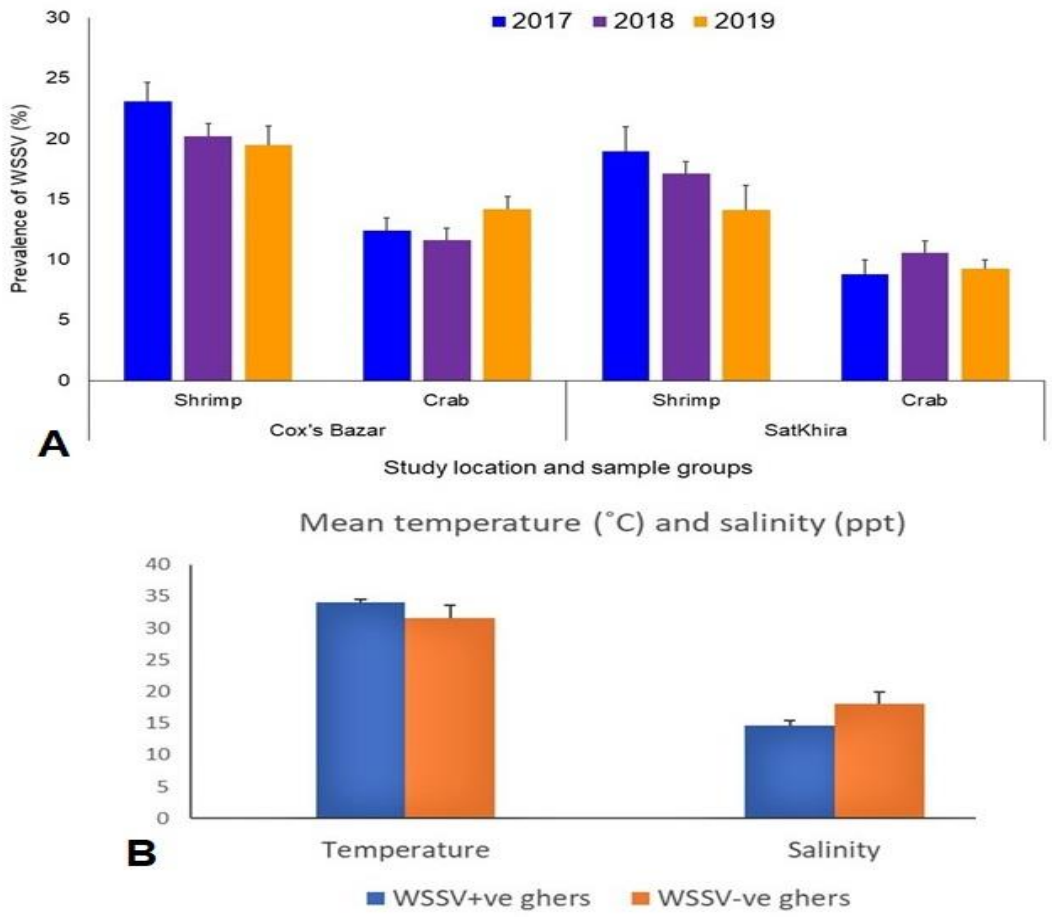

Figure 3.1 WSSV's overall prevalence in Satkhira and Cox's Bazar during 2017-2019. (A). Prevalence of WSSV in shrimps and crabs of two separate coastal regions (Cox's Bazar and Satkhira) (B) Significant difference of mean temperature and salinity between WSSV +ve and WSSV -Ve ghers.

3.4 Viral loads in crustacean samples

Per gram Log load of WSSV in shrimp tissue samples varied from 7.62 (Cox's Bazar) to 12.35 (Satkhira) in the current study. Log load in per gram tissue of crabs ranged from 8.20 (Cox's Bazar) to 10.47 (Satkhira), similarly. Significantly higher ($p < 0.05$, Kruskal Wallis test) viral load (10.48±0.32, SEM) was found in the collected shrimp samples from Satkhira in comparison to that of Cox's Bazar (8.40±0.08, SEM). There was a significant variation in the viral loads observed in crabs, ($p < 0.05$, Kruskal Wallis test) between the study areas

showing higher values of load (9.92±0.56, SEM) in Satkhira when compared to those of Cox's Bazar (8.91±0.72, SEM) (Figure 3.2).

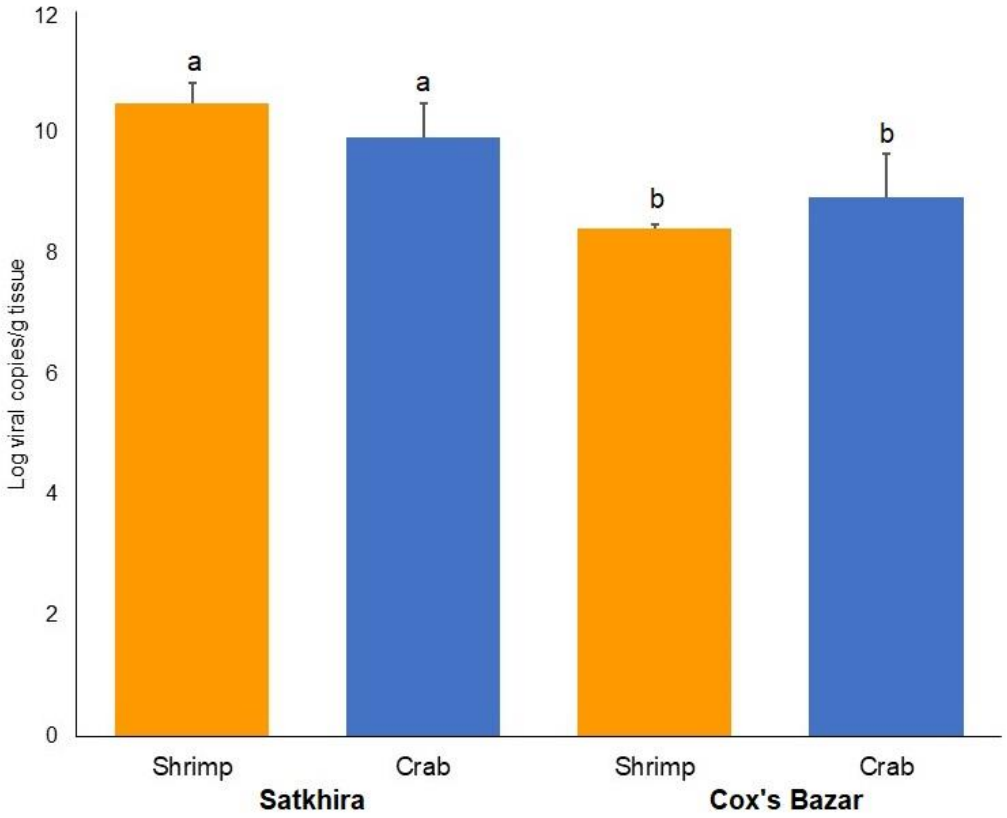

Figure 3.2 Log WSSV copies per gram tissue in shrimp and crab samples of Cox's Bazar and Satkhira. 'Alphabets' point to significant differences in the quantified load of WSSV in the samples collected from both districts ($p<0.05$)

Phylogenetic analysis was done to observe the viral envelope protein VP28 gene-based variations which separated the isolates into two major groups (BD1 and BD2) currently circulating across both of the selected shrimp farming regions of the country. Nucleotide sequences of VP28 gene attained from seven of the sampled crustacean isolates of 2017-2019 along with 17 previously reported reference sequences from Bangladesh (our laboratory) and 10 reference sequences from the NCBI database were applied to create a phylogenetic tree. Two clusters (BD1 and BD2) included 24 of the sequences from Bangladesh which was

mainly related to isolates from India and Vietnam. BD1 and BD2 contained 8 and 16 isolates of WSSV, respectively (Figure 20A). Two of the isolates (MZ383193 and MZ383194) that belonged to phylotype BD1 had 98-100.0% similarity with six VP28 gene sequences stated in the past from Bangladesh (2015 and 2017), and that of two Indian sequences (Figure 3.3A). The isolates of BD1 phylotype made slightly distant branch with sequences of VP28 reported from other countries like China, South Korea and Vietnam. The isolates of BD2 phylotype (MZ383195-198) were observed to be intimately clustered with other VP28 sequences that was formerly reported (2014-2015) to be circulated in Bangladesh (Figure 3.3A).

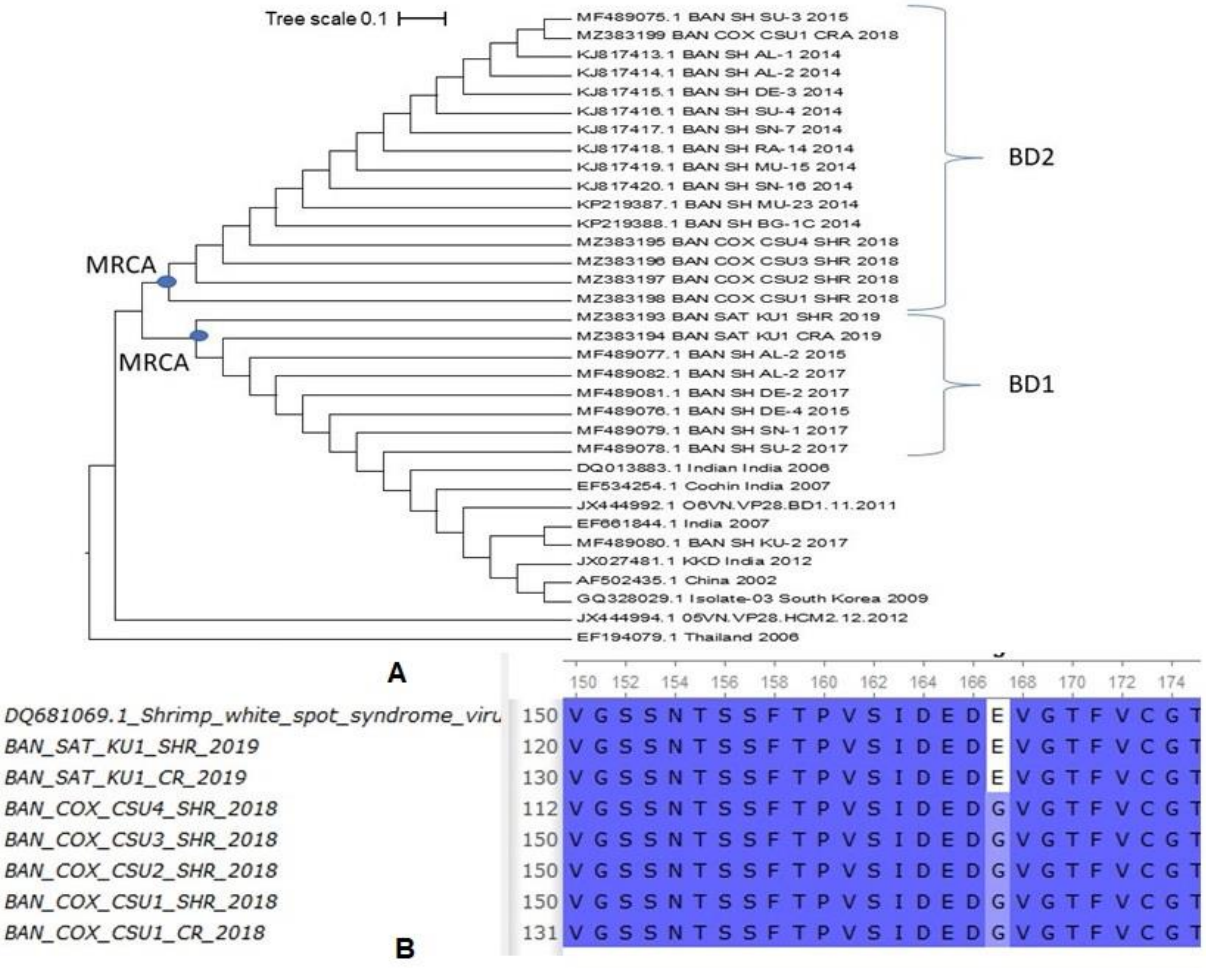

Figure 3.3. VP28 gene-based phylogenetic analysis. (A) Two phylotypes (BD and BD2) are currently circulating across the country. (B) Amino acid (aa) mutations in the VP28 sequences of WSSV.

3.5 Variations in amino-acid mutations of VP28

One VP28 reference sequence was used for the analysis of amino acid (aa) mutations from Thai isolate (GenBank Accession no. AF369029). 104 (69.33%) sequences confirmed aa mutations at 21 positions in the 150 VP28 sequences saved from NCBI GenBank database (including 24 sequences from our laboratory). In all of these mutations, we observed residue position 42 (33.33%), 114 (8%) and 167 (12.67%) as the major mutation sites in VP28 sequences (Figure 3.4). Nevertheless, residue position 167 exhibited glycine instead of glutamic acid (E→G) in 19 VP28 sequences together with 15 from Bangladeshi and four from Egyptian sequences (Figure 3.4). Out of all the Bangladeshi sequences, 10 were taken from former studies of our lab (Hossain et al., 2015, Siddique et al., 2018), and rest of the five (GenBank Accession no. MZ383195-MZ383199) were taken from the this study. Furthermore, in the present study, two VP28 sequences of both shrimp (GenBank Accession no. MZ383193) and crab (GenBank Accession no. MZ383194) isolates from Satkhira contained E at 167 aa position unlike the other five sequences (GenBank Accession no. MZ383199; crab sample, and GenBank Accession no. MZ383195-MZ383198; shrimp samples) of Cox's Bazar with G in that particular residue position (Figure 3.3B).

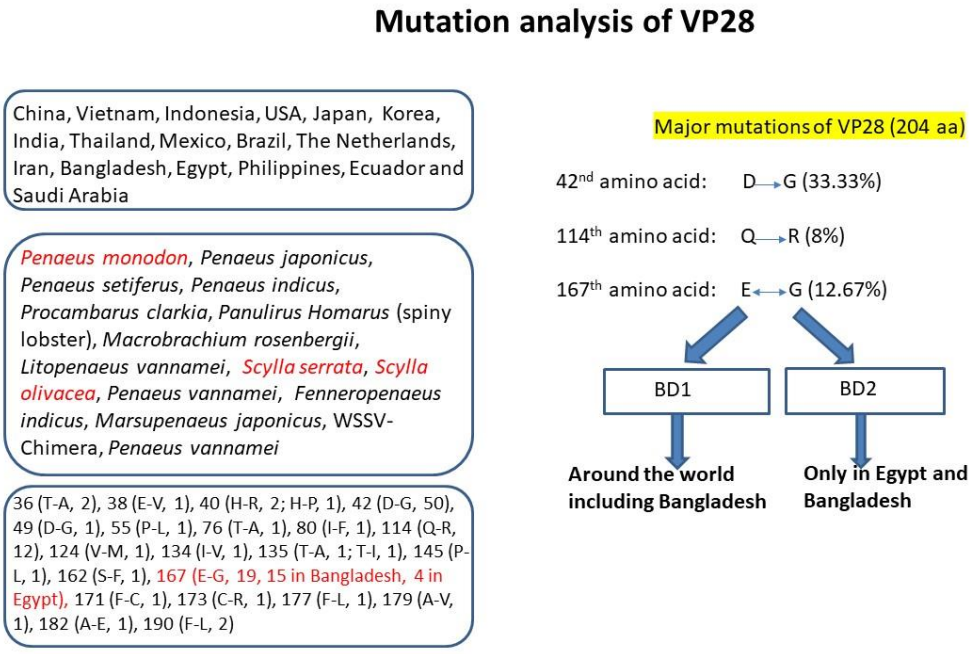

Figure 3.4 Major mutations of VP28 reported from different countries and hosts (our sequences and previously published sequences downloaded from NCBI were used for the analysis).

3.6 Shrimp post larvae mortality rates and lethal time

Statistically significant ($p < 0.05$, Kruskal Wallis test) difference was observed in the mortality of shrimp PL between the control and the experimentally infected groups in all our infection assays of this study. PL of shrimps when treated with WSSV regardless the stated phylotypes exhibited 96.67% mortality at 96 hrs with a dose of 10^8 copies of WSSV per mL of sterile seawater (Figure 3.5, Table 4) in which the death of shrimps were checked in the interval of 6 hrs. In that specific infection assay with three treatments, these young crustaceans set out to decease at 48 hrs and all completely died in 108 hrs while those crustaceans which were in control group survived and did not expire prior to those were sacrificed.

For the relative virulence determination of both the groups with two different doses, it was observed that PL experimentally infected with Dose 1 (0.423×10^9 virus copies per mL)

began to die after 66 hrs of infection assay; and 100% PL mortality occurred within 102 hrs. Nevertheless, the onset of mortality of shrimp PL took place after a 72 hrs challenge with WSSV, while 100% mortality was observed after 108 hrs and 114 hrs of treatment with BD1 and BD2, respectively, after applying Dose 2 (0.423 x 10^7 virus copies per mL) (Figure 3.6, Table 5). Median LT_{50} values of 73 and 75 hrs post infection (hpi), respectively in BD1 and BD2 challenged shrimp PL were noted after the application of Dose 1, and 82 and 84 hpi using Dose 2 (Figure 3.6). Then again, this variation in median LT_{50} between these two groups with both the doses did not show statistical significance ($p > 0.05$). Besides doses and the viral phylotypes, PL's physiological conditions along with their ability to adapt in aquariums may also be considerable factors connected to mortality. Because of these observations, it was postulated that viral load quantification in the experimentally infected PL tissue could be very useful in achieving the target of the study with more accuracy. And considering that point, to quantify the number of copies of WSSV by the use of real-time PCR in the challenged organisms with any particular dose of WSSV was an important component of this research.

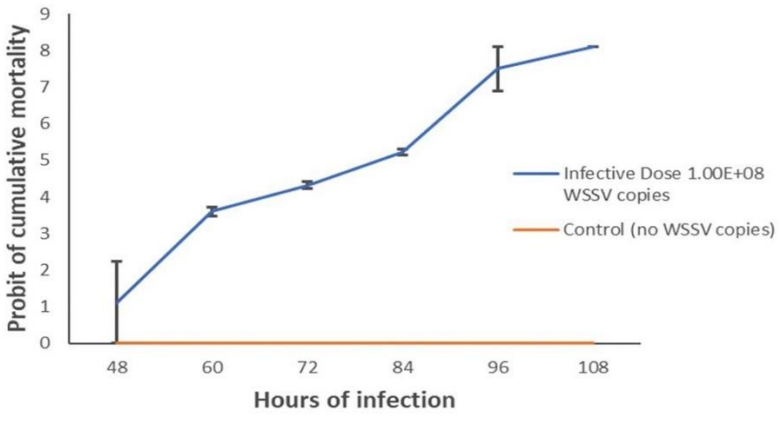

Figure 3.5 Probit of mortality. The Y-axis displays the rate of mortality while the X-axis symbolizes the hrs of infection.

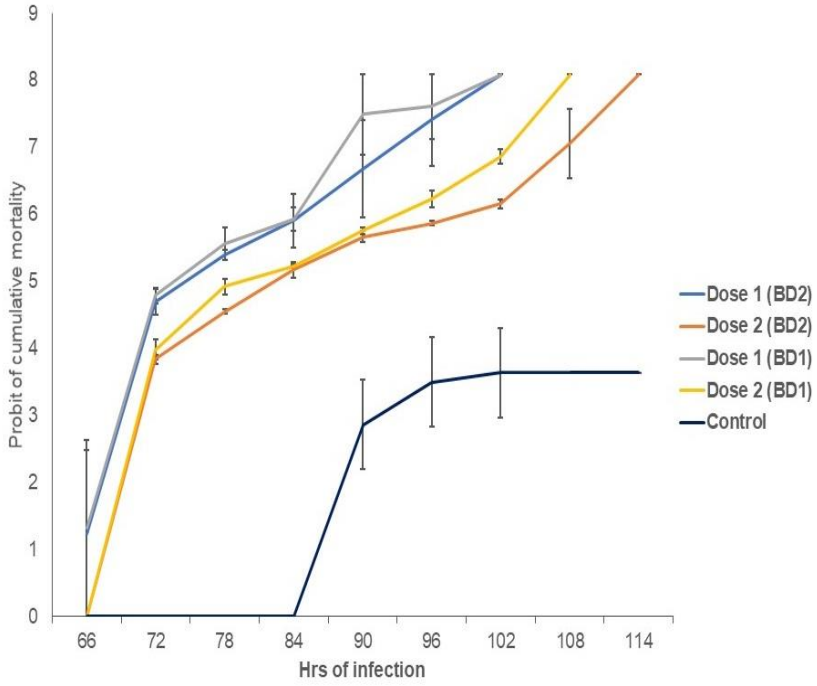

Figure 3.6 Probit of cumulative mortality of shrimp PL after treatment with BD1 and BD2 phylotypes. The Y-axis displays the rate of morality, and the X-axis presents the hrs of infection.

3.7 Quantification of WSSV in challenged shrimp PL

Mean Log WSSV copies were found to be 6.47 and 4.75 per mg of crustacean tissue respectively in treated PL with BD1 and BD2 that were statistically significant ($p < 0.05$, Kruskal Wallis test) with Dose 1 demonstrated in Figure 3.7. In this analysis, mean C_T values of 20.01 and 25.32 were observed in BD1 and BD2 treated PL, respectively (Table 4). Positive amplifications were observed in the challenged samples of both phylogroups, positive controls and standards (Figure 3.8). A wide range of mean Log copy numbers of WSSV in the treated PL was noted in such a particular quantitative analysis, and outcomes indicated specific amplification curves for standards, samples, positive controls and negative controls. Notable fluorescence signal could not be observed for negative control (NTC). C_T

values corresponding to NTC were beyond the index of determination, and C_T values in correspondence to the positive samples in quantitative real-time PCR ranged from 7.19 to 33.48 (Figure 3.8A). A specific amplification product of 148 bp was visualized in agarose gel electrophoresis after qPCR with the qVP28F and qVP28R primers. The standard curve produced was linear from log starting quantity 2 to 9. Mean upper and lower levels of quantified viral load in the shrimp PL challenged with WSSV were observed to be 1.10 x 10^{10} and 3.31 x 10^2 copies of WSSV per mg tissue, respectively (Figure 3.8A and 3.8B).

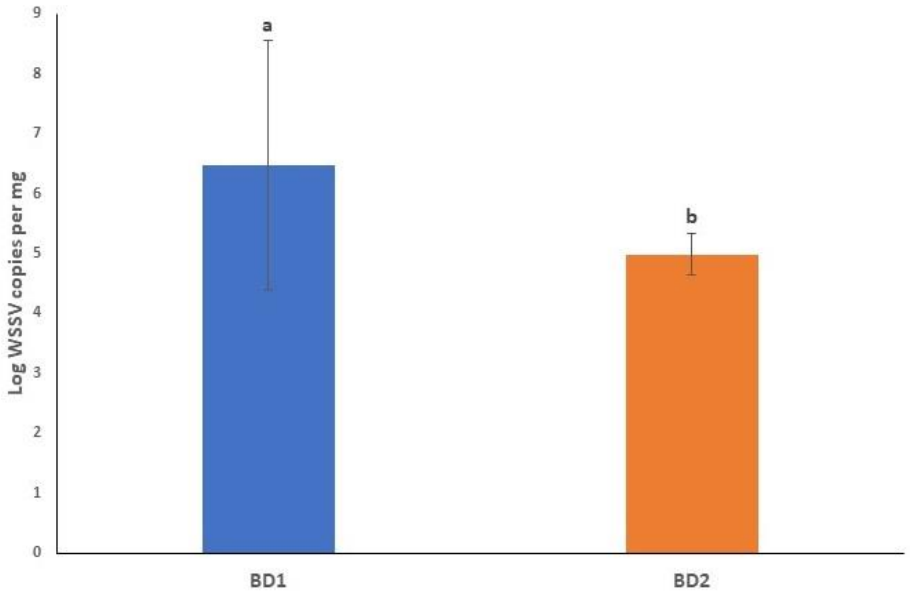

Figure 3.7. Log viral load per mg tissue experimentally infected with BD1 and BD2. Error bars and 'alphabets' represent the standard deviation and significant difference ($p<0.05$), respectively.

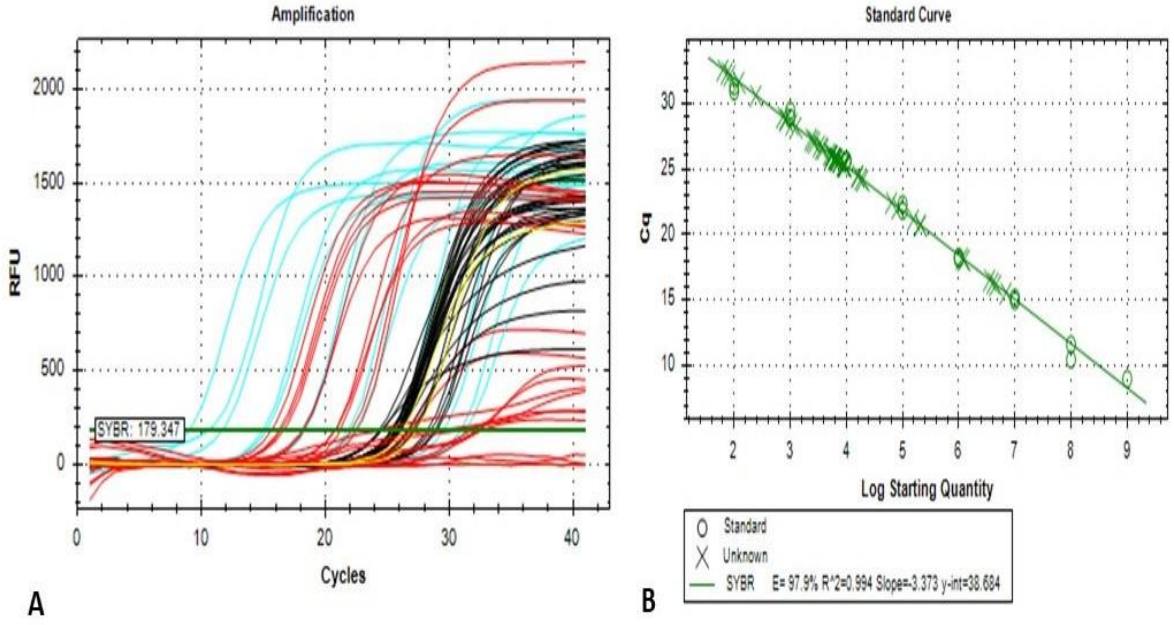

Figure 3.8. (A) Amplification curves targeting VP28 of WSSV and (B) standard curve produced using quantitative real-time PCR. Plasmid samples with known concentrations were used to obtain the standard curve, and copy numbers of unknown samples were calculated comparing Threshold Cycle values (CT) of samples and standards.

3.8 Mortality rate and viral load counts observed from crab infection assay

In the crab infection assay, mortality rate of crabs and viral load counts differed between the treated groups with BD1 and BD2 phylotypes. The LT_{50} and LT_{100} of WSSV challenged crabs varied among the different treatments. On the other hand, no crab was observed to die in the negative control group. Infection assay was conducted for 62 days post infection (dpi) till all crabs died in the treated groups with both phylotypes (BD1 and BD2) (Figure 3.9). The LT_{50} and LT_{100} were 31 and 48 dpi in crabs infected with BD1, and on the other hand, LT_{50} and LT_{100} were observed to be 40 and 62 dpi in the infection assay of crabs treated with BD2, respectively. Mean copy numbers of virus in the crabs treated for infection with BD1 and BD2 were 12.06±0.48 and 9.95±0.37 per g of tissue, respectively (Figure 3.10).

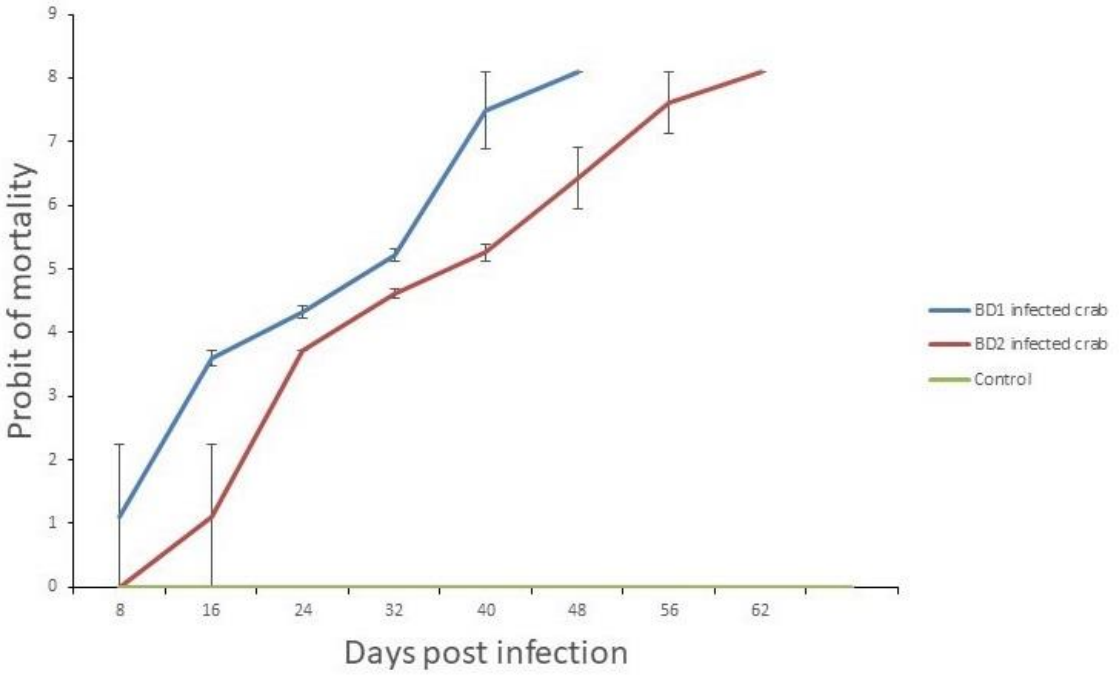

Figure 3.9 Probit of death of WSSV-treated crabs with ingestion method of inoculation applying a dose prepared from BD1 and BD2 infected crab tissue, and WSSV-negative crab tissue (Control). The Y-axis shows the rate of mortality, and the X-axis represents the days post infection.

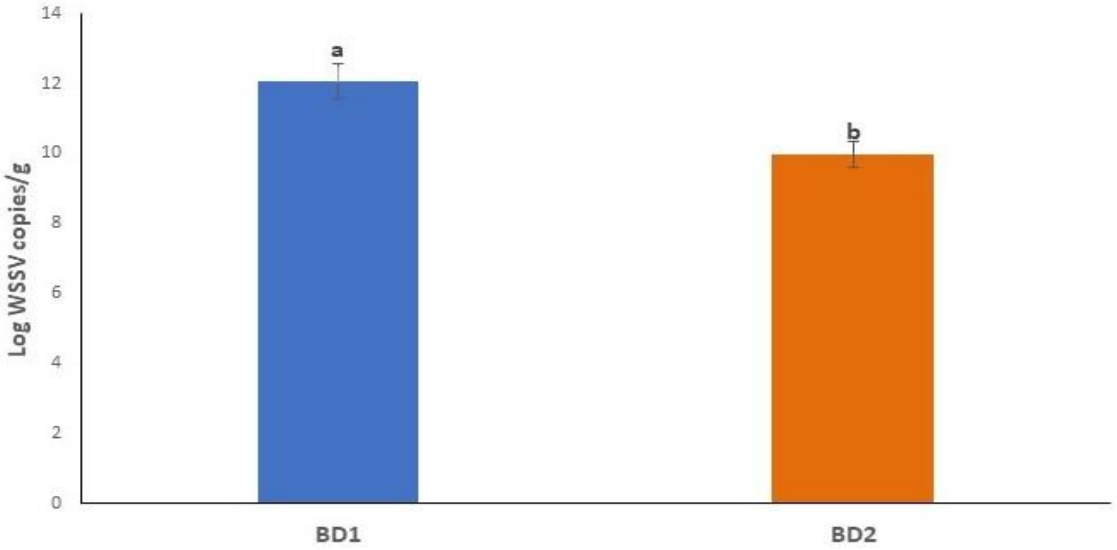

Figure 3.10. Viral load in experimentally infected crabs of both groups. Error bars and 'alphabets' correspond to standard deviation and significant difference ($p<0.05$), respectively.

3.9 Gene expression profiling of immunity genes

For gene expression analysis, two critical immunity genes of shrimps were taken into consideration, penaeidin and lysozyme, which belonged to antimicrobial peptides (AMPs). In the immersion challenge study, expressions of these two immunity genes were observed after 73 hpi. In WSSV-exposed shrimp PL, the average relative expression was comparatively higher for lysozyme than penaeidin in both the groups (Figure 3.11). Meanwhile, it was observed that the average of both of these two genes' relative expressions were at a lower level in BD1 treated PL than in BD2 treated PL (Figure 3.11).

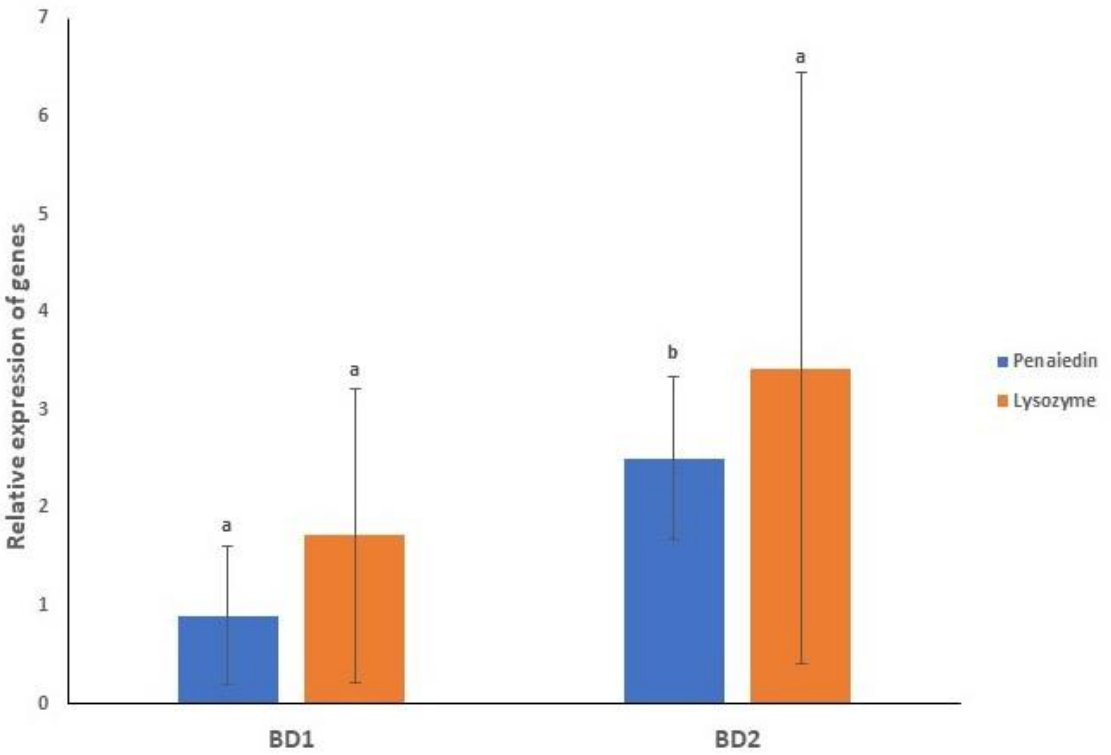

Figure 3.11. Average relative expression of immunity genes (Penaeidin and lysozyme) in experimentally infected shrimp PL with WSSV. Error bars and 'alphabets' correspond to standard error of means and significant differences between the expression of immunity genes, respectively.

3.10 Rab7-VP28 binding affinity

There was lower K_d value observed for the binding affinity of VP28 having glutamic acid at the 167th aa position (1.6E-08) than the other mutated one with glycine on that position (5.1E-08). It might be predicted that the higher the K_d value the lower could be the binding intensity between proteins (Figure 3.12). As of such an *in-silico* attempt for finding out the binding affinity in two distinct complexes, it was projected that the BD1 could create better possibility to bind with host receptor Rab7. Furthermore, prediction outcomes as of DynaMut indicated a rise in molecular flexibility instead of rigidification in VP28 of BD2 over analysing the variation in vibrational entropy (ΔΔSVib ENCoM: 0.030 kcal.mol^{-1}.K^{-1}) (Figure 3.12).

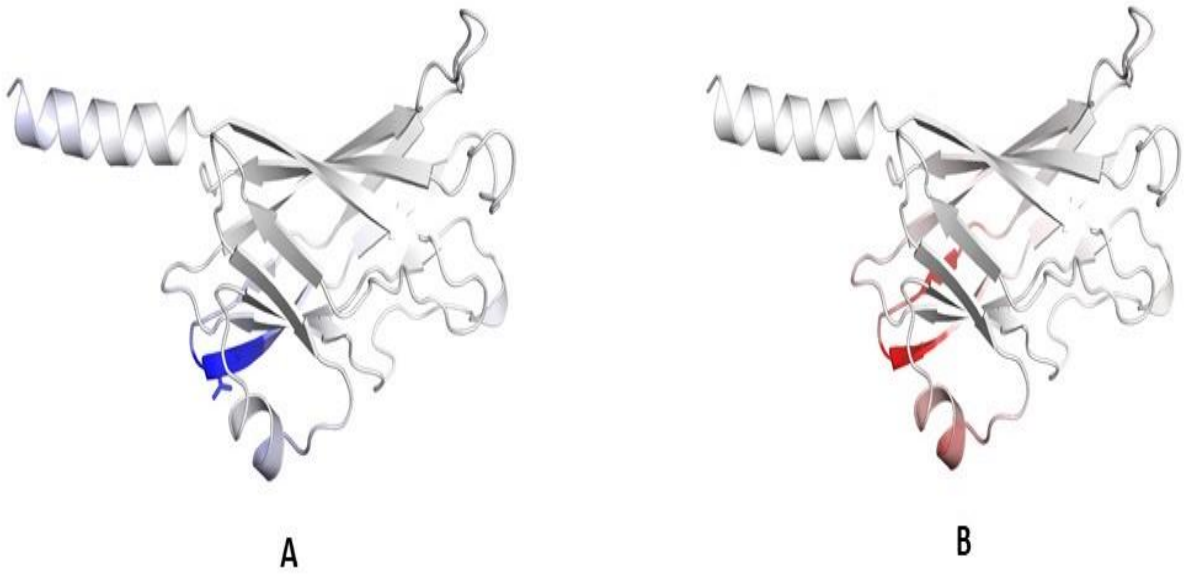

Figure 3.12. A. Glycine to Glutamic Acid signifies rigidification and B. Glutamic Acid to Glycine at 167th amino acid position signifies gaining flexibility using DynaMut web server. 'Blue' signifies a rigidification of the structure (G167E) (Figure 3.12A) and 'Red' represents a gain in flexibility (E167G) (Figure 3.12B).

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Chapter 04:
Discussion

4.0 Discussion

4.1. Prevalence of WSSV and viral loads varied according to geographic locations and crustacean species

Penaeus monodon is among the most prominent, valuable and economically profitable cultivated aquatic organisms in Bangladesh. This crustacean has been known to be severely affected by this deadly virus in all of the nations across Asia that produce shrimps along with Bangladesh (Ayub et al., 2008, Hossain et al., 2015). In this research, the occurrence of illnesses caused by WSSV in crustaceans showed variations in the study regions maintaining considerably greater prevalence of WSD in shrimp ghers of Cox's Bazar when assessed in comparison to Satkhira. The WSSV exposure rate in crabs stayed much lower in comparison to shrimps which could be linked to their disease lenience capacity and carrier position (Molla et al., 2009). Mud crabs are commonly considered to be comparatively more resilient to WSSV unlike shrimps which can keep on carrying WSSV for extended periods not showing signs of illness. In the current research, the WSSV-positivity had been confirmed in crabs of only those ghers in which the shrimps were noticed to be WSSV-positive as well. When *P. monodon* and *Scylla* spp. are together in these improved traditional ghers, the exposure of WSSV to shrimps through horizontal transmission from the carrier crabs could cause WSSV infection in shrimps which may lead to death of shrimps (Tuyen et al., 2014). Therefore, it is quite essential to examine frequently whether these shrimps are getting diseased for the presence of WSSV-carrier crabs to prevent WSSV infections and eliminate the source of the disease.

WSSV had a greater prevalence in the ghers of Satkhira which was reported in an earlier study (Hussain et al., 2015). 23% prevalence of WSSV in the south-west and south-east coast of India was reported in wild crustaceans including *Scylla serrata*, *Squilla mantis*, *Penaeus*

indicus, and *Metapenaeus* spp. (Vaseeharan et al., 2003). Fegan and Clifford (2001) opined that pH, ammonia, salinity, dissolved oxygen, water temperature, and toxins originated from pesticides could be associated with bulk deaths due to WSSV. A substantial impact of water temperature on the WSD and mortality of infected crustaceans were notified in a previous work (Vidal et al., 2001). Analysis of physico-chemical parameters illustrated that *P. monodon* has the ability to tolerate distinct variations in salinity (13-21 ppt), pH (7.8-9.1), dissolved oxygen (2.61-6.06 ppm), and temperature (28.7-34 ˚C). The usual range in a previous study for salinity 15-25 ppt, pH 7.5-8.5, dissolved oxygen more than 4 ppm and temperature 28-32˚C showed similarity in conjunction with the findings of this research (Tendencia and Verreth, 2011). Significant association was noticed between rise in temperature and drop in salinity with the occurrence of WSSV in our selected improved traditional ghers of both Cox's Bazar and Satkhira districts. This might also be due to the impact of these environmental factors on the susceptibility for the crustaceans to get infected and the activities of their immune system. It was reported that sudden decrease in salinity might perhaps increase the mortality rate of shrimp infected by WSSV (Liu et al., 2006; Peinado-Guevara and López-Meyer, 2006). In this current study, the dissolved oxygen also showed considerable association with the presence of WSSV. Regular variations in physicochemical components of the culture pond water get shrimps vulnerable to stress eventually leading to WSD (Zafar et al., 2015, Páez-Osuna et al., 2003). Previous research works had suggested about a critical role of temperature and salinity in causing WSSV infection which influence crustaceans immune response (Páez-Osuna et al., 2003, Le Moullac and Haffner, 2000). Moreover, it was also reported that extreme situations of physico-chemical factors in the culture ponds can restrain innate immunity of shrimps including release of oxygen radicals, decrease in total hemocyte count, phagocytic index and proPO activation (Le Moullac and Hafner, 2000).

In this study, shrimps and crabs contained enormous quantity of WSSV in their tissue. In comparison to the samples of Satkhira, WSSV Log load per gram tissue stayed relatively lower in the samples. However, assessing the quantity of virus in the samples of both the crustaceans, shrimp and crab, no statistically significant variation was observed. The existence of such huge counts of copy numbers of virus dangers all the shrimps being cultured in the ghers setting up a greater risk for WSSV epidemic in the entire region. The current research recommends that quantification of copy numbers of virus in the diseased shrimp is crucial because in the presence of very lightly infected organisms with WSSV in the culture pond can even lead towards outbreak (Siddique et al., 2018). All of the shrimps in a gher could gradually get exposed to WSSV over ingestion or immersion if few shrimps in that facility are affected by the deadly WSSV causing a quick spread of the disease by propagation of WSSV leading to disaster in production (Siddique et al., 2018).

4.2 VP28 based phylogeny analysis showed genetic variance

VP28 based phylogeny analysis indicated that sequences of neighboring countries Bangladesh and India pitched into the main clades (BD1 and BD2). The solitary viruses in Bangladesh demonstrated genetic variance which came down under two separate bunches (BD1 and BD2). WSSV isolates from several nations including India, China, South Korea and Vietnam created such distinct clusters. In this research work, the VP28 (MZ383195-MZ383198) sequenced in 2018 (BD2 phylogroup) demonstrated close genetic relatedness with VP28 sequences of Bangladesh reported earlier (Hossain et al., 2015). Similarly, two VP28 isolates (MZ383193- MZ383194) that were sequenced in 2019 (BD1) exhibited strong ancestral relation to six VP28 isolates which were sequenced in 2015 and 2017 from Bangladesh and with two Indian isolates. Hence, phylogeny analysis presumed that all VP28

sequences of the isolates in the current study (2018 and 2019) were intimately connected to the VP28 sequences stated from Bangladesh in the past (2011-2017), and the outcomes are associated with the former studies (Hossain et al., 2015, Siddique et al., 2018).

VP28 has a major role in recognizing the host cell. WSSV's VP28 is the most critical structural protein for attaching and penetration into the shrimp cells for development of systemic infection (Chang et al., 2010). Mutational analysis of the amino acids indicated that most of the VP28 sequences of WSSV (64%) stated from several geographic regions (together with the seven sequences of this research) experienced mutations at twenty one points. Different previous research works from Bangladesh (Siddique et al., 2018; Hossain et al., 2015) and neighboring countries (Tang et al., 2007, Verma et al., 2013) had reported aa variations in VP28 as well, and the present findings are in accordance with those. In augment, amino acid position 167 exhibited glycine as a substitute of glutamic acid (E→G) in some of the sequences of WSSV's envelope protein VP28 from Bangladesh and Egypt. The distinctive alteration (E→G) at aa position 167 falls between two beta strands, and is considered to be associated with the action for recognizing the receptor (Verma et al., 2013, Siddique et al., 2018). In the isolates of Bangladesh collected in 2014, 2015 and 2018 (Siddique et al., 2018, Hossain et al., 2015), the sole aa mutation at VP28's 167th aa position was found. WSSV-host association starts when VP28 unites with host receptor (*Pm*Rab7). The nucleocapsid of the virus is later moved to the host cell's nucleus where viral genome begins to replicate (Verma et al., 2013). The binding of PmRab7 with the envelope protein VP28 was initially confirmed by Sritunyalucksana et al. (2006). Our prediction outcomes applying DynaMut for the assessment of molecule flexibility provided a feeling that there might be rigidification in binding interactions for VP28 of BD1 and had also produced a sense of probability of firmer binding affinity with the VP28 of BD1. K_d values assist in

presuming that there might be higher binding affinity in Rab7-VP28 complex when shrimps get infected with the BD1 phylotype. The role of mutations in VP28 is also very essential to be studied as this envelope protein being transgenically engineered was used in various research works for the purpose of creating immunity in crustaceans, and as it has the ability to infect by localizing on the epithelial cells of the host. Researchers were also curious on giving attention to designing of drugs by applying molecular docking and simulation works involving VP28 (Chandrika and Puthiyedathu, 2021).

4.3 Mortality rates and viral load counts varied in the crustaceans challenged experimentally

Experimentally infected shrimp PL with dilution containing 10^8 WSSV copies demonstrated a death rate of 97% at 96 hrs regardless of any group, BD1 or BD2. When those PL were challenged with two of the mentioned groups with different loads demonstrated distinguishable variation in mortality patterns. The onset of mortality and the lethal time 50 (LT_{50}) in experimentally infected PL was observed to be inversely proportional to the dilution stock (quick expiry time with the dose relatively less diluted). LT_{50} values for tested PL varied between the phylotypes (BD1 and BD2) and remained elevated in shrimp PL treated with BD2. On the other hand, the average median LT_{50} were observed to be at a lower level in the shrimp PL treated with BD1. Average Log WSSV copy numbers were observed to vary between both the groups of challenged PL holding statistically greater (6.47 per mg tissue) in PL challenged with BD1. In several countries and also in the studies where mud crabs were treated with experimental infection, it was noticed that crabs were carriers and vectors of WSSV (Chen et al., 2000; Gunasekaran et al., 2018).

There was a pilot experiment for virus infectivity assay with crabs in this research, where the

crabs were infected through ingestion method. The findings of the pilot study demonstrated that the crabs treated with BD1 expired earlier than those treated with BD2. Amount of viral loads in all the samples of both groups treated with WSSV exhibited increased load of WSSV in crabs treated with BD1 like the shrimp PL treated with BD1. Other authors have reported variations in amounts of viral loads in their infectivity studies (Durand and Lightner, 2002; Rahman et al., 2008), and perhaps such differences can be explicated by variations in scale of physiological condition and state of host defense and virus replication. It is assumed in our study that hosts affected by BD1 phylotype might contain greater number of WSSV copies when the infection is in a later stage as our experimental results showed higher copy number of WSSV and lower Ct values in BD1 infected samples. However, vulnerability to WSSV might vary between different species of decapods and stage of life (Hameed et al., 2003, Escobedo-Bonilla et al., 2005), nevertheless, application of a recognized dose for various mutated groups of deadly WSSV is crucial to establish the differences. In a previous study where shrimp PL were challenged with immersion technique by the use of an inoculum having known amount of copies of virus, it was indicated that there is requirement of at least five logs of WSSV copies to create symptoms of WSD which could deliver a median LT_{50} of 52 hrs (Escobedo-Bonilla et al., 2005). Gunasekaran et al. (2018) followed different methods for infectivity assays, and it was observed in their study that the ingestion method caused mortality quicker than water-borne technique in case of crabs. Although both crustacean species were treated with two different techniques for infection, the crabs perished later in our experiments. May be, such results came out of the crabs being very hardy species and for their ability to remain as the carrier of virus for a longer period. On the other hand, shrimps died faster after getting infected. This is certainly very important to be taken into consideration that PL of shrimps are not so hardy like crabs to fight with viral infections, and the PL stage is an early stage of life to combat with diseased condition. The cumulative

mortality was noted to be 100% in experimental infection assays with shrimp PL tested with immersion technique or through oral administration at 108 hours post infection with several amount of doses where the LT_{50} for doses, low to high, were observed to be 65, 57 and 50 hpi in the analysis of Escobedo-Bonilla et al. (2008).

4.4 Gene expression of immunity genes differed between phylotypes

To add values in support of the results of infection assays of the present study, immunity related genes Penaeidin (Pen) and Lysozyme (Lyso) were considered for expression profiling in the current study. There are few reports in which the activities of AMPs against virus were described as these are the parts of the innate immunity of shrimps. In two different studies, a synthetic AMP was used in vitro in shrimps inoculated by WSSV where mortality was observed to get lowered for such pretreatment with the synthetic AMP (Dupay et al., 2004). Pen, which is an AMP, expressed in penaeid shrimps mostly recognized to exhibit antibacterial and antifungal activities was found to operate a crucial role in the immune response when these crustaceans got exposed to deadly WSSV (Woramongkolchai et al., 2011). In the shrimp PL groups challenged with WSSV, there were observed differential forms of gene expressions indicating the possibility of transcription due to two extremely important phases of defense, recovery (wound healing) and killing of microbes (Song and Li, 2014; Li et al., 2010; Munoz et al., 2002; Bachere et al., 2000; Kawabata et al., 1996). It also needs to be considered that the cysteine-rich domain of Pen's C-terminal together with the amphipathic form could play an important part as domain for binding pathogenic microorganism. Lyso, another valuable AMP, is participating in the battle of host against attacking microbes (Liu et al., 2017; Xing et al., 2009; Sotelo-Mundo et al., 2003). In an earlier research work with blue shrimp (*Litopenaeus stylirostris*), upregulation of Lyso was observed in WSSV-infected shrimps, indicating its participation in shrimp's innate immunity

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(Mai and Wang, 2010). This study also holds the observation that the studied immunity genes' average relative expressions were at a lower level in the BD1 challenged PL group in which faster 100% mortality and higher amount of WSSV copies were observed than the other group challenged with BD2.

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Chapter 05:
Conclusion

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5.0 Conclusion

This current research examined the prevalence of WSSV phylotypes circulating across Bangladesh, and special emphasis was given to assess their virulence properties. There was significant variations observed between hosts, geographic locations of the ghers (Cox's Bazar vs Satkhira districts), and also during the periods of study (2017 to 2019). However, with an increasing number of samples from ghers including different other locations of crustacean aquaculture could illustrate more about the overall scenario of the occurrence of this deadly virus in Bangladesh. However, we have a future plan to extend this work throughout the country recruiting a larger samples group and geoclimatic conditions. Nevertheless, lower average median LT_{50}, quicker 100% mortality and higher viral load in crustaceans challenged with BD1 phylotype were observed in comparison to those with BD2 phylotype in the infection assays. The average relative expression of AMPs, penaeidin and lysozyme, was at a low level in BD1 challenged group when equated with BD2. Thus, the results of this study hints that WSSV's virulence may possibly vary depending on the VP28 gene based phylotypes (BD1>BD2). Still, more research works targeting the immunity of economically valuable crustaceans are essential to comprehend the vivid biological systems of these invertebrates when infected by this deadly WSSV. Although we are still in an initial stage to pull a final conclusion on the virulence properties of these phylotypes, the findings of this research work could be useful for the safeguards in farms against this deadly WSSV, and might create opportunity to reduce the enormous financial losses in shrimp farming of Bangladesh every year.

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Chapter 06:
References

6.0 References

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Appendices

Appendix-I

>BAN COX CSU1 CR 2018

ATTGCTGTATTTATTGTGATTTTTAGGTATCACAACACTGTGACCAAGACCATCGAAACCCACACAGGCAATA

TCGAGACAAACATGGATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGCTACTTCAAGATGAC

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CACCTTTGAGGGAACATTCAAGGTGTGGAACAACACATCAAGAAAGATCAACATCACTGGTATGCAGATGGT
GCCAAAGATTAACCCATCAAAGGCCTTTGTCGGTAGCTCCAACACCTCCTCCTTCACCCCCGTCTCTATTGATGA
GGATGGAGTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTACCGCCGGTGGAAATCTT
TTCGACATGTACGTGCACGTCACCTACTCTGGCACTGAGACCGA

>BAN COX CSU1 SHR 2018

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CAAAGGCCTTTGTCGGTAGCTCCAACACCTCCTCCTTCACCCCCGTCTCTATTGATGAGGATGGAGTTGGCACC
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CGTCACCTACTCTGGCACTGAGACCGAGTAAGA

>BAN COX CSU2 SHR 2018

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>BAN COX CSU3 SHR 2018

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AGCGACACCTTGGGCAAAATCAAGATCCGCAATGGAAAGTCTGATGCACAGATGAAGGAAGAAGATGCGGA
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GGCCGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACCTTTGAGGGAACATTCAAGGTGTGGAACAACACA
TCAAGAAAGATCAACATCACTGGTATGCAGATGGTGCCAAAGATTAACCCATCAAAGGCCTTTGTCGGTAGCT
CCAACACCTCCTCCTTCACCCCCGTCTCTATTGATGAGGATGGAGTTGGCACCTTTGTGTGTGGTACCACCTTTG
GCGCACCAATTGCAGCTACCGCCGGTGGAAATCTTTTCGACATGTACGTGCACGTCACCTACTCTGGCACTGA
GACCGAGTAA

>BAN COX CSU4 SHR 2018

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ACTGGTATGCAGATGGTGCCAAAGATTAACCCATCAAAGGCCTTTGTCGGTAGCTCCAACACCTCCTCCTTCAC
CCCCGTCTCTATTGATGAGGATGGAGTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTA
CCGCCGGTGGAAATCTTTTCGACATGTACGTGCACGTCACCTACTCTGGCACTGAGACCGAA

>BAN SAT KU1 CR 2019

GCTGTATTTATTGTGATTTTTAGGTATCACAACACTGTGACCAAGACCATCGAAACCCACACAGGCAATATCGA
GACAAACATGGATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGCTACTTCAAGATGACTGAT
GTGTCCTTTGACAGCGACACCTTGGGCAAAATCAAGATCCGCAATGGAAAGTCTGATGCACAGATGAAGGAA
GAAGATGCGGATCTTGTCATCACTCCCGTGGAGGGCCGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACC
TTTGAGGGAACATTCAAGGTGTGGAACAACACATCAAGAAAGATCAACATCACTGGTATGCAGATGGTGCCA
AAGATTAACCCATCAAAGGCCTTTGTCGGTAGCTCCAACACCTCCTCCTTCACCCCCGTCTCTATTGATGAGGA
TGAAGTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTACCGCCGGTGGAAATCTTTTCG
ACATGTACGTGCACGTCACCTACTCTGGCACTGAGACCGAGTA

>BAN SAT KU1 SHR 2019

AACACTGTGACCAAGACCATCGAAACCCACACAGGCAATATCGAGACAAACATGGATGAAAACCTCCGCATTC
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AATCAAGATCCGCAATGGAAAGTCTGATGCACAGATGAAGGAAGAAGATGCGGATCTTGTCATCACTCCCGT
GGAGGGCCGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACCTTTGAGGGAACATTCAAGGTGTGGAACA
ACACATCAAGAAAGATCAACATCACTGGTATGCAGATGGTGCCAAAGATTAACCCATCAAAGGCCTTTGTCGG
TAGCTCCAACACCTCCTCCTTCACCCCCGTCTCTATTGATGAGGATGAAGTTGGCACCTTTGTGTGTGGTACCA
CCTTTGGCGCACCAATTGCAGCTACCGCCGGTGGAAATCTTTTCGACATGTACGTGCACGTCACCTACTCTGGC
ACTGAGACCGAGTAG

The reported WSSV sequences of this study have been submitted to the GenBank database under the accession numbers MZ383193 to MZ383199.

Appendix 2

Solutions and Reagents used

Preparations of the stock solutions used in this work are given below: (all the working solutions used in this work were prepared from the stock solutions).

5 M NaCl

29.22 g of NaCl was dissolved in distilled water to a final volume of 100 ml. The solution was autoclaved and stored at room temperature.

1 M KCL

7.444 g of KCl was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

1 M MgCl2

20.33 g of MgCl2 was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

1 M MgSO4 24.648 g of MgSO4 was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

1 M glucose 19.817 g of Glucose was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

0.5 M EDTA 186.1 g of Na2EDTA.2H2O and 20.0 g of NaOH pellets were added and dissolved by stirring to 800 ml distilled water on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

3 M sodium acetate

40.81 g of Na2 (CH3COOH).H2O was dissolved in 80 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with distilled water and the solution was sterilized by autoclaving. It was stored at 4°C.

TAE buffer

242 g of tris-base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) was taken and distilled water was added to the mixture to make 1L. 1X concentrated TAE buffer was made by adding 10 ml 50X TAE buffer with 490 ml distilled water and stored at room temperature.

Ethidium bromide solution

10 µl of ethidium bromide was dissolved in 100 ml TAE buffer to make a final concentration of 20 mg/ml and stored at 4°C in the dark

Gel loading buffer

Ingredients	**Amount (g/L)**
Sucrose	6.7
Bromophenol blue	0.04
Distilled water	Upto 1 L

Wizard® SV Gel and PCR Clean-Up System. Catalog No. A9282

Reagents	**Purpose**
Membrane Binding Solution	Help in binding of PCR product
SV Minicolumn	For Binding of PCR product
Collection Tube	For collection of flow throw
Membrane Wash Solution	For washing purposes
Nuclease-Free Water	For elution of the purified DNA from the GD column
SYBR Green master mix	

Wizard®Plus SV Minipreps DNA Purification System. Catalog No.A1460 (For Plasmid DNA)

Reagents	**Purpose**
Cell Resuspension Solution	For the resuspension of pelleted cells
Cell Lysis Solution	For lysis of cells
Alkaline Protease Solution	For the degradation of cellular proteins
Neutralization Solution	For Nutralizaton of AlkilineProease
Spin Column	For Binding of the plasmid DNA molecules
Collection Tube	For collection of flothrow
Wash Solution	For washing purposes
Nuclease-Free Water	For elution of the plasmid DNA from the GD column

Maxwell® 16 Total DNA Purification Kit. Catalog No. AS1050

Reagents	
Maxwell® 16 RNA Cartridges	Lysis Buffer
DNA Dilution Buffer	Clearing Agent (CAA)
Nuclease-Free Water	Mercaptoethanol, 97.4%
Clearing Columns	Collection Tubes

Plungers	Elution Tubes

ATPTM Genomic DNA Mini Kit (Blood/Culture Cell/Bacteria) Catalog No. AGB100/AGB300

Reagents	**Purpose**
GT Buffer	For the resuspension of pelleted cells
GB Buffer	For lysis of cells
GD Column	For Binding of the DNA molecules
Collection Tube	For collection of flothrough
W1 Buffer	For washing purposes
Wash Buffer (ethanol added)	For washing purposes
Elution Buffer	For elution of DNA from the GD column

New England Biolab's RNA extraction kit

ProtoScript® II First Strand cDNA Synthesis Kit

Appendix 3

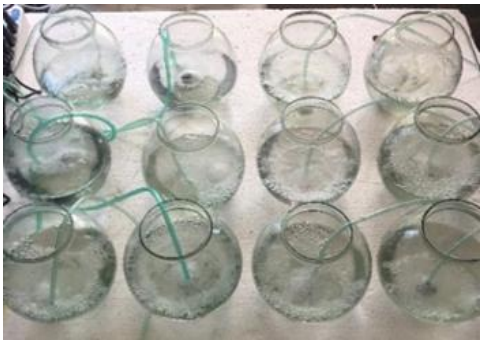
Instruments & Apparatus

Instruments	Origin
ABI Prism 3130 Genetic Analyzer	Applied Biosystem, USA
AlphaImager HP System Versatile Gel Imaging	Cell Bioscience, USA
Autoclave, Model no: HL-42AE	Hirayama corp, Japan
Microcentrifuge (temperature controlled)	Sigma, USA
Class II Microbiological Safety Cabinet	Nuaire, USA
Electric balance, Scout, SC4010	Shimadzu, Japan
Freezer (-30°C)	Liebherr, Germany
Horizontal Gel ElctrophoresisApparatus Hl-SET	CBS Scientific, UK
Incubator	Japan
Microcentrifuge	Mikro20, Germany
Microcentrifuge tube	Eppendorf, Germany
Micropipettes	Eppendorf, Germany
Microwave oven, Model: D90N30 ATP	Butterfly, China
NanoDrop 2000	Thermo Scientific, USA
Thermal Cycler	Biometra , Germany; Veriti 96 well Thermal Cycler, USA; ProFlex PCR System, USA
pH meter	Hanna Instruments
Power pack	Toledo, Germany
Refrigerator (4 °C)	Vest frost
Room temperature horizontal shaker	Gerhardt, Germany
Sterilizer, Model no: NDS-600D	Japan
Water bath, Model:SUM	England
-80º C Freezer	Nuaire, USA
MaxwellR 16 Instrument	Promega, USA
Real-time PCR system 7500	Applied Biosystems, Foster City, CA, USA
Dry block Heating/Cooling thermostat	Grant – bio, England
Aquariums	
Aerators	
Thermometre	Hanna Instruments
Salinity meter	Hanna Instruments
DO meter	Hanna Instruments

Appendix 4

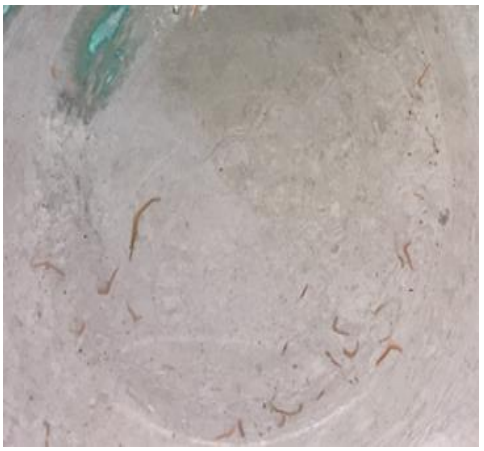
Figure S1. (a) Shrimp PL collected from Meghna Shrimp Hatchery, (b) Aerated small aquariums for rearing of PL, (c) PL swimming inside small aquariums and (d) Dead PL at the bottom of the aquariums

(a)



(b)

(c)



(d)

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Figure S2. Crabs for treatment with WSSV including the negative control group

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Table S1. Probit of deaths in three treatments (T1, T2, T3) with WSSV-positive inoculum and in negative control (C1, C2, C3)

Hours	T1	T2	T3	C1	C2	C3
48	-	-	3.36	-	-	-
60	3.72	3.36	3.72	-	-	-
72	4.33	4.16	4.48	-	-	-
84	5.39	5.13	5.13	-	-	-
96	8.09	6.28	8.09	-	-	-
108	8.09	8.09	8.09	-	-	-

Table S2. Cumulative mortality of shrimp PL challenged with two doses (T1G, T2G and T3G mean three treatments with Dose 1 prepared from BD2; T1G2, T2G2, and T3G2 mean three treatments with Dose 2 from BD2; while T1E, T2E and T3E mean three treatments with Dose 1 prepared from BD1, T1E2, T2E2, and T3E2 mean three treatments from Dose 2 prepared from BD1)

Hours	T1G	T2G	T3G	T1G 2	T2G 2	T3G 2	T1E	T2E	T3E	T1E 2	T2E 2	T3E 2	C1	C2	C3
66	36	0	0	0	0	0	54	0	0	0	0	0	0	0	0
72	144	90	180	45	54	36	144	126	180	36	81	54	0	0	0
78	234	216	252	126	117	108	234	216	306	135	189	180	0	0	0
84	288	216	342	198	170	180	288	270	324	216	216	207	0	0	0
90	324	270	360	261	288	261	360	324	360	270	288	279	0	17	0
96	360	306		288	297	188		342		306	315	333	18	17	24
102		360		315	306	324		360		351	342	351	18	23	24
108				342	333	360				360	360	360	18	23	24
114				360	360								18	23	24

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Table S3: C_T values and mean WSSV copies from the quantitative real-time PCR of crab ingestion challenge pilot experiment (E and G prefixes of sample IDs represent samples challenged with BD1 and BD2).

ID	CT	CT	CT	CT mean	CT SD	Mean WSSV copies per gram
G1	35.137949	34.823678	35.00544	34.98902249	0.158	$2.56x10^{9}$
G2	32.150755	32.057954	32.362684	32.19046422	0.156	$1.62x10^{10}$
G3	33.342938	33.252047	33.744374	33.44645309	0.262	$7.04x10^{9}$
G4	31.815467	31.729114	31.687042	31.74387431	0.065	$2.06x10^{10}$
G5	33.810558	33.733273	34.251021	33.93161766	0.279	$4.67x10^{9}$
G6	31.842761	31.401744	32.359593	31.86803256	0.479	$1.89x10^{10}$
E1	26.871992	26.670694	26.648922	26.73053588	0.123	$7.10x10^{11}$
E2	31.953898	25.723672	26.080568	27.91937927	3.499	$3.02x10^{11}$
E3	27.238455	27.081096	27.249458	27.18966927	0.094	$5.42x10^{11}$
E4	24.774002	26.646636	26.707501	26.04271329	1.099	$1.07x10^{12}$
E5	23.993787	23.685243	23.821705	23.83357793	0.155	$4.61x10^{12}$
E6	23.51504	23.944252	24.26711	23.90880065	0.377	$4.27x10^{12}$

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Appendix 5

microorganisms

MDPI

Article

Circulating Phylotypes of White Spot Syndrome Virus in Bangladesh and Their Virulence

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Abstract: White Spot Syndrome Virus (WSSV) has emerged as one of the most prevalent and lethal viruses globally and infects both shrimps and crabs in the aquatic environment. This study aimed to investigate the occurrence of WSSV in different ghers of Bangladesh and the virulence of the circulating phylotypes. We collected 360 shrimp (*Penaeus monodon*) and 120 crab (*Scylla* sp.) samples from the south-east (Cox's Bazar) and south-west (Satkhira) coastal regions of Bangladesh. The VP28 gene-specific PCR assays and sequencing revealed statistically significant ($p < 0.05$, Kruskal–Wallis test) differences in the prevalence of WSSV in shrimps and crabs between the study areas (Cox's Bazar and Satkhira) and over the study periods (2017–2019). The mean Log load of WSSV varied from 8.40 (Cox's Bazar) to 10.48 (Satkhira) per gram of tissue. The mean values for salinity, dissolved oxygen, temperature and pH were 14.71 ± 0.76 ppt, 3.7 ± 0.1 ppm, 34.11 ± 0.38 °C and 8.23 ± 0.38, respectively, in the WSSV-positive ghers. The VP28 gene-based phylogenetic analysis showed an amino-acid substitution (E→G) at the 167th position in the isolates from Cox's Bazar (referred to as phylotype BD2) compared to the globally circulating one (BD1). Shrimp PL artificially challenged with BD1 and BD2 phylotypes with filtrates of tissue containing 0.423×10^9 copies of WSSV per mL resulted in a median LT50 value of 73 h and 75 h, respectively. The in vivo trial showed higher mean Log WSSV copies (6.47 ± 2.07 per mg tissue) in BD1-challenged shrimp PL compared to BD2 (4.75 ± 0.35 per mg tissue). Crabs infected with BD1 and BD2 showed 100% mortality within 48 h and 62 h of challenge, respectively, with mean Log WSSV copies of 12.06 ± 0.48 and 9.95 ± 0.37 per gram tissue, respectively. Moreover, shrimp antimicrobial peptides (AMPs), penaeidin and lysozyme expression were lower in the BD1-challenged group compared to BD2 challenged shrimps. These results collectively demonstrated that relative virulence properties of WSSV based on mortality rate, viral load and expression of host immune genes in artificially infected shrimp PL could be affected by single aa substitution in VP28.

Keywords: VP28; WSSV; phylotypes; real-time PCR; viral load

check for updates

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