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 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 X 10^9 copies of WSSV per mL resulted a median LT50 value of 73 hrs and 75 hrs, 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 hrs and 62 hrs 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 was lower in 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, real-time PCR, viral load, apoptosis

Introduction

Shrimp aquaculture is one of the major earning sources in many countries including Bangladesh and plays a vital role in enlightening community advancement, food security, employment opportunities and poverty reduction [1,2]. In Bangladesh, shrimp aquaculture provides livelihood to around 85 million people (mostly coastal people), serves as the second most foreign currency-earning source which contributes about 5% to national GDP [3,4]. Black tiger shrimp (*Penaeus monodon*) contributes 26% to the total aquacultural production in Bangladesh while crabs contribute 6% [5]. Studies suggested that *P. monodon* can adapt to wide range of salinity from 4 to 40 ppt, dissolved oxygen from 4 to 7 ppm, temperature from 25 °C to 32 ˚C, and pH from 7.5 to 9 [6,7]. The mud crab, *Scylla* spp. are distributed widely throughout the Indo-Pacific region [8]. Recently, mud crab (*Scylla olivacea*) farming has an increasing trend in the coastal areas of Bangladesh due to their higher disease resistant capacity and market values [9]. Among the marine crustaceans, crabs are supposed to be less vulnerable to the effects of climate change and deterioration of water quality. To date, more than 98 species have been found as hosts or carriers of WSSV [10], and of them, mud crabs have been considered to be a particularly potential threat to shrimp farms because of their carrier status [10-12]. Moreover, mud crabs could suppress viral replication by inducing apoptosis of hemocytes [13].

The WSSV is one of the major threats to the shrimp industry over the past two decades globally. This is a very fast reproducing, wide spreading and highly virulent crustacean pathogen [14,15]. Studies have shown the widespread pathogenicity of WSSV among many marine crustaceans, including shrimp, crayfish and crab [13,16]. The outbreak of WSSV

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depends on the interactions among the pathogen, host and environment [17], and thus, the interaction between WSSV and the hosts have been a research focus in recent years. This virus can be transmitted both horizontally and vertically [12,18], and once an outbreak of WSSV occurs, it wipes out entire population in many aquatic farms within a few days [14]. Infection of the WSSV on shrimp is characterized by a rapid mortality up to 100% within 7–10 days [19]. Up to now, there is no effective method to prevent the shrimp from WSSV infection. Appropriate protective management are enormously important for reducing WSSV infections in shrimp farms [20]. In Bangladesh, eggs are hatched in the hatcheries from mother shrimps collected from the Bay of Bengal, and shrimp post larvae (PL) from these hatcheries are distributed throughout the country. PL traders directly catch and sell to shrimp farmers as well. Breeding of WSSV-resistant varieties should be the most effective method to solve the virus disease problem [15]. However, comprehensive study regarding the physicochemical parameters of ghers (shrimp ponds) of Cox's Bazar and Satkhira districts of Bangladesh, and identification of circulating WSSV in those areas are lacking.

The circular genome of the WSSV is approximately 275 nm in length and 120 nm in width with tail-like appendages at one end, and composed of five known major structural proteins: VP28, VP19, VP26, VP24 and VP15 [19,21]. Studies on WSSV viral proteins have demonstrated that VP28 and VP19 are associated with the virion envelope [21,22] while VP26 acts as a tegument protein linking the two nucleocapsid-associated proteins VP24 and VP15 to the envelope [17,23]. The VP28 is required for WSSV entry into host cells by endocytosis, cell-to-cell infection and virus propagation [22]. Moreover, this envelop protein plays significant role in initiating the WSSV infection in shrimp [22]. The VP28 gene of the WSSV is suggestively involved in endosome escape through its interaction with host Rab7 [24], and has been identified as a potent target for dsRNA treatment in comparative studies [25]. Previous analyses of strain variability reported that competitive fitness depends on the size of the

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genome [26,27], however, these studies are far from enough to illustrate the mechanisms of WSSV pathogenesis. The on-going mutations in the structural proteins like VP28 could explain most phenotypic variance of WSSV for certain traits [15,28]. Siddique et al. (2018) confirmed a mutation at amino acid position 167 of VP28 gene of WSSV in Bangladesh with glycine instead of glutamic acid on that position. However, the virulence properties of different phylotypes of WSSV till remained unknown. Therefore, this study investigated the impact of physicochemical parameters on the prevalence of WSSV, virulence properties of circulating phylotypes of WSSV based on mortality observation and viral load count in shrimp PL artificially infected with two circulating phylotypes of WSSV.

Materials and methods

Sampling and measurement of physicochemical parameters

Shrimp (*P. monodon*) and crab (*Scylla* sp.) 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 which are situated in the South-East and South-West coastal regions of Bangladesh (Figure S1). In this study, samples were collected from 20 ghers during the period when farmers reported presence of WSD in the area (five from Cox's Bazar and fifteen from Satkhira). A total of 360 shrimps and 120 crabs were grossly collected after farmers complain regarding death of the crustaceans in their farming ghers (shrimp ponds) during 2017 to 2019. Data on temperature, pH, dissolved oxygen and salinity were collected from the study ghers.

DNA extraction

After initial screening considering symptoms of disease, tissue DNA was extracted from the collected samples by automated DNA extraction system (MaxWell 16® Tissue DNA Purification kit; AS 1030, Promega, USA), according to manufacturer's instruction [29].

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Challenged shrimp PL tissue were collected in sterile 1.5 ml microfuge tubes and mashed into fine particles with glass rod prior to DNA extraction. DNA concentration and purity were measured by Nano-Drop 2000 (Thermo Scientific, USA) [20].

Conventional and Quantitative real-time PCR (qPCR) assay

The extracted DNA underwent to a conventional PCR assay to amplify the VP28 gene using GoTaq 2 X Hot Start Colorless Master Mix (Promega, USA) with forward and reverse primers [29,30]. The conventional PCR reactions included denaturation at 95 ˚C for 50 minutes, annealing at 55 ˚C for 30 seconds, extension at 72 ˚C for 45 seconds, and repeated for 30 cycles with a final extension of 5 minutes at 72 ˚C. Amplified PCR products were separated and visualized on 1.0% agarose gel in TAE buffer with ethidium bromide staining. Following electrophoresis, the bands were photographed under UV light (Figure S2A).

The real-time qPCR was performed with primer pair WSSV-q28F 5′-TGTGACCAAGACCATCGAAA-3′ and WSSV-q28R 5′-CTTGATTTTGCCCAAGGTGT-3′ following previously developed methods with some modification (recombinant plasmid based standard instead of purified PCR product based standard) [29,31]. The recombinant plasmid containing VP28 gene (TOPO TA Vector having complete CDS of VP28 gene as an insert) was gel purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The standard was prepared from serial dilution of purified recombinant plasmid in a linear logarithmic scale of 1.0 X 10^9 to 10^2 copies per reaction. In brief, all qPCR reactions were 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 primers and variable amount of each template DNA. Thermal cycling parameters were set for an initial denaturation step at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s for DNA denaturation with subsequent annealing and

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extension at 53 °C for 30 s. Melt curve analysis was also performed to differentiate the specific amplicon from primer dimer formation or amplification of other non-specific product. Moreover, the qPCR products were also electrophoresed in agarose gel to nullify the presence of spurious amplicon. The present experiment was conducted with duplicates replication to quantify the viral load. WSSV load per gram of tissue sample was calculated according to the following equation:

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 assess the reproducibility of the standard curve, standard reactions were performed three times independently including duplications of each reaction. The real-time PCR data were analysed by using 7500 software, version 2.0.6 (Applied Biosystems, Foster City, CA, USA). The data were analysed by using the statistical program Microsoft Excel 2020 and presented as mean ± SD. Standard deviation of the viral load per reaction was considered during the viral load calculation. For relative virulence study, viral copy numbers in the challenged 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 S2B).

Sequencing of VP28 protein, phylogenetic and mutation analyses

Conventional PCR amplicons were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, USA), and the seven (shrimp = 5; crab = 2) purified PCR products were exposed to an automated dideoxy cycle sequencing reaction using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems®, USA) according to manufacturer's instruction [20]. 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 employed to remove all ambiguous, and low-quality sequences [32].

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The raw sequence data were assembled through SeqMan version 7.0 (DNASTAR, Inc., Madison, WI, USA) and the assembled sequences were compared with other entries from NCBI GenBank [33] with BLAST [34] search to disclose the identification and matching with VP28 gene of WSSV.

Using Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 for the larger datasets [35], the VP28 gene sequences, amplified from seven isolates, were aligned with each other, and with relevant reference sequences from our previous study (n = 17) and NCBI GenBank database (n =10), with >90% taxonomic identity. A maximum-likelihood tree was generated with the Tamura-Nei evolutionary model [35,36]. Nodal confidence in the resulting phylogenetic relationships was assessed using the bootstrap test (1000 replicates) [37]. Seven VP28 sequences of WSSV different isolates of shrimp and crab and 143 reference sequences of VP28 retrieved from GenBank were subjected to a multiple alignment through MAFFT [38], and some adjustments were made by manual editing. Repeat units of each isolate were annotated using Geneious Prime (Trial Version), and aligned against a reference sequence for amino-acid (aa) variability score counting [39,40].

Experimental infection

P. monodon post larvae (PL) were collected from Meghna Shrimp Hatchery, Cox's Bazar, Bangladesh with a length of 1-2 cm. WSSV-free breeder wild-caught mother shrimps' eggs were used for hatching these post larvae in the hatchery. Highly infected black tiger shrimp tissue was used for preparing WSSV inoculum. Tissue below carapace from affected shrimp was minced and homogenized in sterile sea water. Supernatant was collected after centrifugation at 8515 X g for five minutes and filtered through 0.45 µm membrane. Stock was diluted to prepare infective dose containing 10^8 copies per mL. Blank inoculums were prepared using the same steps from a WSSV-negative shrimp sample. For in-vivo challenge, we performed previously established 'immersion technique' [41] as waterborne inoculation [42].

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The experimental groups (n = 180) were challenged by immersion technique in aerated glass jars with WSSV solutions in three treatments. Using the WSSV-negative inoculums, aerated jars were set to treat the control group (n = 180). The PL were fed with commercially available artificial feed once a day at the rate of 10% body weight. Mortalities were checked after every six hrs, and the presence of WSSV was checked by conventional PCR (Figure S2C).

Virulence determination assay was performed in separate experiments with three treatments of shrimp PL (n=360) infected with inoculums containing same copy number of both phylogroups (BD1 and BD2). A control group was maintained with inoculum prepared from the WSSV-negative tissue. Thus, inoculums were prepared using above mentioned procedure from tissue having same copy numbers of virus (4.27×10^9) from both groups and added in the small aquariums for treatments. Infective doses consisted of filtrates from tissue of both groups containing 0.423×10^9 and 0.423×10^7 copies of WSSV per mL in sterile sea water. Temperature of the experimental tanks' water ranged between 28-29 ˚C while the salinity and dissolved oxygen were 18 ppt and 5-6 ppm, respectively throughout the experiment. Time dependent mortality rates were measured by counting the number of dead PL in every six hrs interval. DNA from challenged PL (infected and control) were extracted and tested for viral load estimation by real-time PCR. C_T values and copy number of WSSV obtained from different samples were compared.

An infection trial was conducted on mud crabs through ingestion method with three treatments and one control containing 5 crabs in individual tanks. Crabs of the treated groups were fed with infected crab tissue (Day 1) and trash fish (from Day 2) while crabs of the control group were given beef liver from the first day (Gunasekaran et al. 2018). Tissue containing both groups of WSSV (1.0×10^9) were used to feed the crabs of the experimental groups. Standard experimental conditions were similar with the shrimp PL virulence experiment other than the salinity which was maintained at a higher level (28-30 ppt).

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RNA extraction and preparation of cDNA

RNA was extracted from the challenged PL at moribund stage using QIAgen's QIAamp Viral RNA mini kit according to the protocol of manufacturer. The purity and concentration of the extracted RNA were assessed by Nanodrop-2000 spectrophotometer. The extracted RNA was reverse-transcribed to cDNA using New England Biolab's cDNA kit (PhotoScript II First Strand cDNA Synthesis Kit).

Gene expression analysis

Real-time PCR was used for the analysis of expression of two immunity genes of shrimps (Penaeidin and Lysozyme). Quantitative PCR was run 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 using New England Biolab's Luna Universal qPCR master mix according to the protocol of manufacturer (The primers used are presented in Table S3). Average-fold difference in gene expression was analysed by comparative delta C_T method [43]. Housekeeping gene Beta Actin was used for normalization that provided the C_T values as internal (endogenous) control. Control treatments were inoculated with inoculum prepared from WSSV-negative tissue, and the samples were considered for gene expression if there was any amplification. Delta C_T has been calculated by deducting C_T value of endogenous control from the gene of target, and finally, mean delta C_T was calculated from this standardised delta C_T value. Delta delta C_T was calculated with reference to control by deducting mean delta C_T of control from mean delta C_T of the gene of target. Changes in average-fold gene expression in challenged PL was calculated to $2^{-\text{delta delta } C_T}$ values.

Observation of binding affinity of VP28 and its receptor protein Rab7

Web server PRODIGY was used to observe the binding affinity of receptor protein Rab7 and WSSV envelope protein VP28 of both groups from Bangladesh (Vangone and

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Bonvin, 2015; Xue et al., 2016)). VP28 with glycine at its 167th amino acid position and VP28 with glutamic acid at 167th amino acid position were used for docking with Rab7 of *P. monodon* by HADDOCK (High Ambiguity Driven protein-protein DOCKing) (van Zundert et al., 2016). For protein-protein docking, active residues (Verma et al., 2013) were produced from both molecules. Prior to docking, the Rab7 sequences were downloaded from NCBI and the Homology modelling of Rab7 protein was done using SWISS-MODEL and validated further by developing Ramachandran plots (Biasini et al., 2014). PROGIDY uses interactors from both proteins for producing binding affinity (ΔG) and the dissociation constant (K_d) values. Moreover, DynaMut web server was used to observe the impact of this mutation to the stability of the VP28 (Rodrigues et al.,2018).

Statistical analysis

The salinity, temperature, dissolved oxygen and pH were compared to WSSV grouped as present and absent. In case of salinity, temperature and dissolved oxygen, non-parametric independent sample tests were performed, and for pH, parametric independent sample test was done. Null hypothesis of the distribution of physicochemical parameters same across categories of WSSV was retained for dissolved oxygen and pH, and rejected for salinity and temperature. When no significant interactions exist between time and isolate, the probit model has the form: Probit (x) = α + β time + γ isolate where α is the intercept, β is the rate of probability change per unit change of time (for a constant isolate), and γ is the rate of probability. Eta test statistic was used to study the associations between WSSV (nominal variable) and the physicochemical parameters (scale variables). Additionally, the mean prevalence of WSSV among different regions was calculated using SPSS (SPSS, Version 23.0, IBM Corp., NY USA) using above mentioned tests [44]. Final values for average-fold differences in gene expression were tested for significance at 5% level using t-test.

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Results

Prevalence of WSSV and physicochemical parameters in the study ghers

The overall prevalence of WSSV in both crustacean population (shrimp and crab) between the study areas (Cox's Bazar and Satkhira) and over the study periods (2017-2019) differed significantly ($p < 0.05$, Kruskal Wallis test). The average prevalence of WSSV during 2017 to 2019 in shrimp and crabs 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 1A). By comparing the year wise prevalence of WSSV in the shrimp between the study areas, highest prevalence was recorded in the shrimp ghers of both Cox's Bazar (23.11%) and Satkhira (18.96%) in 2017, and WSSV infection rates in shrimp population gradually decreased thereafter in 2018 and 2019 (Figure 1A). Conversely, the prevalence of WSSV in crab population was found to be highest in the ghers of Cox's Bazar (14.16%) in 2019 and Satkhira (10.54%) in 2018 (Figure 1A). Physicochemical parameters of the ghers showed that the value 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 shrimp 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 1, Table S1). Comparing the physicochemical parameters across the study ghers, we found higher water temperature in WSSV-positive ghers compared to WSSV-negative ghers. Likewise, the WSSV-negative ghers also had higher salinity than WSSV-positive ghers (Figure 1B).

Detection of WSSV in *Scylla olivacea*

One of the novel hallmark findings of this study is the detection of WSSV in the mud crabs (*Scylla olivacea*) in the samples of both regions, Cox's Bazar and Satkhira. Appearance of 643 bp PCR product confirmed the presence of WSSV in the crabs (Figure S2A).

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Viral loads in circulating phylotypes of WSSV differed in crustacean samples

In the current study, WSSV Log load per gram of shrimp tissue ranged from 7.62 (Cox's Bazar) to 12.35 (Shatkhira) (Figure 2). Likewise, WSSV Log load per gram crab tissue ranged from 8.20 (Cox's Bazar) to 10.47 (Satkhira). On an average, shrimp samples from Satkhira had significantly higher ($p < 0.05$, Kruskal Wallis test) viral load (10.48±0.32, SEM) than that of Cox's Bazar (8.40±0.08, SEM). The viral load in crab samples also varied significantly ($p < 0.05$, Kruskal Wallis test) in the study areas keeping higher load (9.92±0.56, SEM) in Satkhira compared to samples from Cox's Bazar (8.91±0.72, SEM) (Figure 2).

The VP28 gene-based phylogenetic analysis divided the isolates into two major phylotypes (BD1 and BD2) which are currently circulating across the country. Nucleotide sequences obtained from seven crustacean isolates according to VP28 gene sequencing (2017-2019) along with 17 previously reported reference sequences (2014-2015) from Bangladesh (our lab) and 10 reference sequences retrieved from NCBI database were used to generate a phylogenetic tree. Two clusters (BD1 and BD2) contained 24 of the sequences isolated from Bangladesh that mostly related to isolates from India and Vietnam. The phylotype BD1 and BD2 contained 8 and 16 isolates of WSSV, respectively (Figure 3A). Two isolates (MZ383193 and MZ383194) belonged to phylotype BD1 had 98-100.0% similarity with six VP28 gene sequences reported previously from Bangladesh (2015 and 2017), and that of two Indian sequences (Figure 3A). The isolates of BD1 phylotype formed slightly distant branch with VP28 gene sequences reported from other countries like China, South Korea and Vietnam. On the other hand, the isolates of BD2 phylotype (MZ383195-198) were found to be closely clustered with other VP28 sequences that was previously reported (2014-2015) to be circulated in Bangladesh (Figure 3A).

Variations in amino-acid mutations in VP28 of WSSV

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For amino acid (aa) mutation analysis, we used one VP28 reference sequence from Thai isolate (GenBank Accession no. AF369029). Out of 150 VP28 sequences retrieved from NCBI database (including 24 sequences from our lab), 104 (69.33%) sequences showed aa mutations at 21 positions. Among these mutations, residue position 42 (33.33%), 114 (8%) and 167 (12.67%) were found as the major mutation sites in the VP28 sequences of WSSV (Figure S3). However, residue position 167 showed glycine instead of glutamic acid (E→G) in 19 VP28 sequences including 15 from Bangladeshi and four from Egyptian sequences (Figure S4). Of the Bangladeshi sequences, 10 were obtained from previous studies from our lab [20,29], and rest of the five (GenBank Accession no. MZ383195-MZ383199) from the current study. Moreover, in the current study, two VP28 sequences of both shrimp (GenBank Accession no. MZ383193) and crab (GenBank Accession no. MZ383194) isolates from Satkhira had E at 167 residue position unlike the five sequences (GenBank Accession no. MZ383199; crab sample, and GenBank Accession no. MZ383195-MZ383198; shrimp samples) of Cox's Bazar having G in that position (Figure 3B).

Shrimp post-larvae mortality rates and lethal time differed between phylotypes

The survival of shrimp post-larvae (PL) between control and infected groups was statistically significant ($p < 0.05$, Kruskal Wallis test) in all of our experiments of the current study. Shrimp PL when challenged with confirmed WSSV irrespective of the mentioned phylotypes showed a mortality rate of 96.67% at 96 hrs with a dose of 10^8 copies of WSSV per mL of sterile sea-water (Figure 4) in which the mortality observation was done in every 6 hrs. In that particular infection assay with three treatments, shrimps started to die at 48 hrs and lasted till 108 hrs while shrimps in the control group did not die before those were sacrificed. By comparing the virulence assay between challenged doses, we found that shrimp PL challenged with Dose 1 (0.423×10^9 virus copies per mL) started to die after 66 hrs of

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challenge, and 100% mortality of PL occurred at 102 hrs after challenge with both phylotypes. However, using Dose 2 (0.423 x 10^7 virus copies per mL), the onset of shrimps PL mortality started at 72 hrs of challenge, and 100% mortality was found after 108 hrs and 114 hrs of challenge with BD1 and BD2, respectively (Figure 5, Figure S2C). Average median LT_{50} values were observed to be 73 and 75 hrs post infection (hpi), respectively in BD1 and BD2 challenged shrimp PL with Dose 1, and 82 and 84 hpi with the Dose 2 (Figure 5). However, this difference in median LT_{50} between the two phylotypes with both doses was not statistically significant ($p > 0.05$). Beside the factors, doses and viral phylotypes, shrimp PL's physiological conditions and capacity to adapt in aquariums could also be the factors engaged in mortality. For that reason, viral load count in the infected PL tissue might help in revealing the study goal more accurately. And from that perspective, quantification of virus using real-time PCR assay was a major part of this study.

Quantitative detection of WSSV in challenged shrimp PL

Mean Log viral copy numbers were 6.47 and 4.75 per mg of tissue respectively in BD1 and BD2 treated PL, which was statistically significant ($p < 0.05$, Kruskal Wallis test) with Dose 1 (Figure 6). In this study, we found an average C_T value of 20.01 and 25.32 in BD1 and BD2 challenged PL, respectively (Table 2, Figure S2B). Figure 7 shows the positive amplifications in samples challenged with both phylogroups, positive controls and standards. In this quantitative assay, an extensive range of mean viral Log load in challenged shrimp PL was observed, and results showed amplification curves were specific for samples, standards, negative controls and positive controls. No noteworthy fluorescence signal was noted for the negative control (NTC). C_T values for NTC were outside determination index, and C_T values in all positive samples had quantitative index in qPCR ranging from 7.19 to 33.48 (Figure 7A). Standard qPCR using the qVP28F and qVP28R primers revealed a single amplicon of 148 bp

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after agarose gel electrophoresis, indicating a specific amplification product. The standard curve generated was linear from log starting quantity 2 to 9. The mean upper and lower quantification limits in challenged shrimp PL were 1.10 x 10^{10} and 3.31 x 10^{2} WSSV copies per mg tissue, respectively (Figure 7A and 7B).

Crab mortality rates and viral load counts differed in treated crabs

The LT_{50} and LT_{100} for infected crabs varied among the treatments while no crab died in the control group. Experiment was run till 62 days post infection (dpi) as long as all crabs died in the experimental groups challenged with both phylotypes (BD1 and BD2) (Figure 8, Figure S5). The LT_{50} and LT_{100} were 31 and 48 dpi in BD1 challenged crabs, and LT_{50} and LT_{100} were 40 and 62 dpi for BD2 challenged crabs, respectively. Mean viral loads in the crabs challenged with BD1 and BD2 were 12.06±0.48 and 9.95±0.37per g of tissue, respectively (Figure 9).

Gene expression profiling of immunity genes in both the infected groups

In the current study, two important immunity genes of shrimps were considered for expression analysis, penaeidin and lysozyme, both of which are antimicrobial peptides (AMPs). Gene expression was observed after 73 hpi in this immersion challenge study. Figure 11 provides the average relative expression of penaeidin and lysozyme after exposure of shrimp PL to both the circulating phylotypes showing comparatively higher expression of lysozyme than penaeidin in both groups. While comparing the expression in the challenged groups, it was found that average relative expressions of these two genes were lower in BD1 challenged PL than in BD2 challenged PL (Figure 10).

Rab7-VP28 binding affinity

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Lower K_d value was observed for the binding of VP28 with glutamic acid at the 167th position (1.6E-08) than the other mutated one with glycine on that position (5.1E-08). The higher the K_d value the lower might be the strength of binding between proteins (Figure S6). From this *in silico* approach towards getting the binding affinity in two different complexes, it was predicted that the BD1 might have more chance to bind with the receptor protein Rab7 of *P. monodon*. Moreover, prediction results from DynaMut showed an increase in molecular flexibility instead of rigidification in VP28 of BD2 through analysing the difference in vibrational entropy (ΔΔSVib ENCoM: 0.030 kcal.mol^{-1}.K^{-1}) (Figure S6).

Discussion

Penaeus monodon is considered as one of the most important commercially cultured aquatic species in Bangladesh. This crustacean species has been badly affected by WSSV in all of the shrimp-producing countries in Asia, including Bangladesh [20,45]. In this study, the prevalence of WSSV infections in crustacean population (shrimps and crabs) varied in study areas keeping significantly higher prevalence of this disease both in shrimp ghers of the Cox's Bazar district compared to Satkhira. Compared to shrimp population, the WSSV detection rate in crabs remained much lower which might be associated with their disease tolerance capacity and carrier status [9]. Unlike shrimps, mud crabs are generally believed to be highly tolerant to WSSV, and keep on infected for longer periods without symptoms of disease infected for long periods of time without signs of disease. In this study, the WSSV-positive crabs were found in those ghers only where the shrimps were also WSSV-positive. Rouf et al. informed in their study that the mud crab species caught from these coastal regions of Bangladesh is *Scylla olivacea* [46]. They confirmed it by genetic analysis of the partial sequences of one mitochondrial gene, 12S rRNA, in *Scylla* spp. collected from the coastal regions of Bangladesh, their morphological characteristics and morphometric ratios. However, if *P. monodon* and

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Scylla spp. are co-cultured, shrimps may become more vulnerable to WSSV infection because shrimps can be infected through horizontal transmission of the virus from the crabs [47]. Thus, it is very important to study regularly whether these shrimps are getting diseased from horizontal gene transfer from carrier crabs to control WSSV infections from shrimp ghers and eradicating the disease sources. In a previous study, Hossain et al. reported higher prevalence of WSSV infections in the shrimp ghers of Satkhira district of Bangladesh [20]. The presence of WSSV was 23% in wild captured crustaceans from the south-west and south-east coast of India which included *Scylla serrata*, *Squilla mantis*, *Penaeus indicus*, and *Metapenaeus* spp. [48]. The physicochemical parameter analysis revealed that *P. monodon* can survive in a wide range of salinity (13-21 ppt), pH (7.8-9.1), dissolved oxygen (2.61-6.06 ppm), temperature (28.7-34 ˚C). Therefore, temperature and salinity were found to be changed very little both in WSSV-positive and WSSV-negative shrimp ghers. In a previous study, it was found that the acceptable range of salinity, pH, dissolved oxygen and temperature can be 15-25 ppt, pH 7.5-8.5, dissolved oxygen more than 4 ppm and temperature 28-32˚C corroborating with our results [6]. We found a significant association between increase in temperature and decrease in salinity with presence of WSSV in shrimp ghers of both Cox's Bazar and Satkhira districts. In this study, dissolved oxygen values also had significant association in the prevalence of WSSV. Frequent fluctuations in physicochemical factors like pH, temperature, dissolved oxygen make shrimps susceptible to stress which ultimately can lead to disease [49,50]. Studies suggested that temperature and salinity play a very important role in WSSV infection affecting the immune response of the crustaceans [50,51].

In the current study, shrimp and crab samples had huge viral loads in tissue. Remarkably, WSSV Log load per gram of shrimp and crab tissues remained much higher in samples of Satkhira district compared to Cox's Bazar. However, comparing the viral load counts in both shrimp and crab samples, we did not find any significant differences. The

presence of such high viral load risks all the shrimps in the ghers as well as the adjacent ghers creating a possibility for WSSV outbreak in the whole area. This study suggests that load determination is essential because shift in temperature due to any environmental reason can lead to outbreak if there is even lightly WSSV infected crustacean in the water body [52]. Siddique et al. (2018) reported that if few shrimps in a gher are infected with WSSV, other shrimps might get exposed over ingestion or immersion, resulting in a rapid spread of the disease leading to production disaster [29].

Phylogenetic analysis showed that VP28 sequences of Bangladesh and India fell into the major clades (phylotype BD1 and BD2). In Bangladesh, the isolated viruses showed genetic divergence falling under two different clusters (BD1 and BD2). These different clusters consisted of WSSV samples from other countries including India, China, South Korea and Vietnam. In this study, the VP28 isolates (MZ383195- MZ383198) sequenced in 2018 (BD2 phylogroup) showed closest genetic relatedness with previously reported VP28 isolates of Bangladesh sequenced in 2014 [20]. Likewise, two VP28 isolates (MZ383193- MZ383194) sequenced in 2019 (BD1 phylogroup) showed closest ancestral relation to six VP28 isolates sequenced in 2015 and 2017 from Bangladesh and two Indian isolates. Thus, from the phylogenetic tree, it can be assumed that all of the isolates sequenced in the present study (2018 and 2019) were quite closely related to the VP28 sequences reported from Bangladesh in the previous years (2011-2017), and the result correlates with the previous works [20,29].

VP28 is one of the most important structural proteins of WSSV responsible for systemic infection and found to be crucial in cell recognition, attaching and penetration into the shrimp cells [53]. The amino-acid (aa) mutational analysis showed that majority of VP28 sequences (64%) of the WSSV reported from different geographical locations (including the seven sequences of the current study) underwent to showed mutations at 21 positions. Several earlier studies from Bangladesh [20,29] and neighbouring countries [54,55] also reported aa variations

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in VP28 of WSSV, and the current results are in line with these reports. In addition, residue position 167 showed glycine instead of glutamic acid (E→G) in Bangladeshi and Egyptian VP28 sequences. In both shrimp and crab isolates from Satkhira district of Bangladesh, the unique mutation (E→G) at position 167 that falls between two beta strands of protein that are thought to be involved in receptor recognition [29,55]. The exclusive aa mutation at residue position 167 of VP28 were also reported in the isolates of Bangladesh collected in 2014, 2015 and 2018 (Siddique et al., 2018, Hossain et al., 2015). VP28 fuses with host protein (PmRab7) which is the beginning of the virus-host relationship, and the viral nucleocapsid is then transported to the nucleus of host cell where the replication of the viral genome starts (Verma et al., 2013). Sritunyalucksana et al (2006) first mentioned that Rab7 protein of penaeid shrimp is involved in binding an envelope protein of WSSV known as VP28. Our prediction results using DynaMut for molecule flexibility analysis impression that there could be rigidification in binding interactions for VP28 of BD1, and had also generated a sense of possibility of stronger binding affinity with the VP28 of BD1. Kd values help to presume that there could be higher binding affinity in Rab7-VP28 complex when shrimps are infected with the BD1 phylotype. As transgenically engineered VP28 had been used in studies to build innate immunity in shrimp for its capacity to localize on host epithelial cells and attention has been given to drug designing using molecular docking and simulation studies (Chandrika and Puthiyedathu, 2021), the role of mutations in VP28 are crucial to be reflected.

Infection assay of shrimp post larvae (PL) with dilution containing 10^8 WSSV showed 97% mortality rates at 96 hrs of challenge irrespective of challenge with any group. Mortality patterns of PL also showed variation when exposed to different loads when challenged with both groups of WSSV. The onset of death and lethal time 50 (LT50) in the experimental PL was found inversely proportional to the dilution stock (earlier death time with less diluted stock). The mean LT50 values for the challenged PL differed between the phylogroups (BD1

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and BD2) of WSSV, and remained higher in BD2 challenged shrimp PL. The average median LT50 remained lower in case of BD1 challenged shrimp PL. Mean Log viral copy numbers were found to differ between both groups challenged PL keeping statistically higher (6.47 per mg tissue) in BD1 treated PL. Mud crabs were found to be carriers and vectors of WSSV in different countries and used for virus infectivity experiments in studies (Chen et al., 2000; Gunasekaran et al., 2018). In our study, crabs were infected through ingestion, and it was found that crabs infected with BD1 died earlier than the ones challenged with BD2. The viral loads in all infected samples showed higher copy numbers in BD1 challenged crabs like BD1 challenged PL. The differences in viral load have also been stated by other authors [56,57], and can possibly be elucidated by differences in degree of virus replication, physiological state, and defence response of the host. Higher WSSV copies and lower Ct values in BD1 challenged samples indicated that this phylotype of WSSV might contain more virus copies at the later stage of infection. There are indications that susceptibility to WSSV may differ between life stages, species and different decapods [58,59], however, the use of a known dose of different phylotypes of WSSV is critical to demonstrate these differences. Immersion challenge to shrimp PL with inoculum of known virus content showed that a minimum of five logs of WSSV copies is necessary to establish disease, and produced a LT50 of 52 hrs [59]. Ingestion method for crab infections have been followed in the study of Gunasekaran et al. (2018) and it was found that in case of crabs the ingestion method resulted faster deaths than water-borne method. Although shrimps and crabs were challenged using two different methods of infection, crabs died later in our study. It may be because the crabs are hardy species and carry virus for a long time, and on the other hand, shrimps die quickly within 3-10 days after infection, and it is also of utmost important to consider that we infected the post-larvae of shrimps which are not so resilient to diseases as those are in early stage of life. Cumulative mortality was observed in shrimp infection assays using immersion or per os inoculation to be 100% at 108 hpi with

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different doses and the LT50 of low to high doses were 65, 57 and 50 hpi in a study conducted by Escobedo-Bonilla et al. (2006).

Gene expression profiling of Penaeidin and Lysozyme were performed to support the findings of infection assays of the current study. Penaeidin (PEN) is an antimicrobial peptide explicitly observed in penaeid shrimp which are commonly known to show antibacterial and antifungal actions were reported to perform a potential part in antiviral immunity of shrimps exposed to WSSV (Woramongkolchai et al., 2011). It was observed that in *P. monodon* PL challenged groups there was a differential patterns of gene expressions, which suggested that transcription could be due to two stages of protection mechanism, killing of microorganisms and wound healing (Kawabata et al., 1996; Bachere et al., 2000; Munoz et al., 2002; Li et al., 2010; Song and Li, 2014). PEN's C-terminal cysteine-rich domain with its amphipathic shape may perform as domain for binding pathogen. Lysozyme (lyso) is another important antimicrobial peptide (AMP) that is involved in the host resistance arrangement of invading microorganisms (Sotelo-Mundo et al., 2003; Xing et al., 2009; Liu et al., 2017). In a previous study with blue shrimp (*Litopenaeus stylirostris*), lysozyme was found to be upregulated in WSSV-infected shrimp, suggesting its involvement in the innate immune response of shrimp to WSSV (Mai and Wang 2010). In the current study, the average relative gene expressions of penaedin and lysozyme in both infected groups expressed at a low level in the BD1 challenged PL where 100% mortality were quicker producing more virus copies.

Conclusions

The current study investigated the prevalence and virulence properties of circulating WSSV in Bangladesh. The prevalence of WSSV was found to differ significantly according to hosts (i.e., shrimp and crabs), geographic locations of the ghers (Cox's Bazar vs Satkhira districts), and also during the periods (2017 to 2019). The *in vivo* infection assay of the shrimp

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PL with BD1 phylotype showed an earlier LT_{50} and LT_{100} and higher viral load compared to those challenged with BD2. The AMPs, penaeidin and lysozyme expression was lower in BD1 challenged group compared to BD2. The findings of the present study revealed that the relative virulence properties of the WSSV could vary depending on the VP28 gene based phylotypes (BD1>BD2). However, extensive studies on the immune system of commercially important crustaceans are required to understand the vibrant biological systems of host crustaceans infected with different groups of WSSV. Although it is still early to draw a conclusion on the virulence of these phylotypes, the results of the present study would be worthwhile for taking precautions in shrimp farms against WSSV and may shed new light to mitigate the huge economic loss every year in shrimp farming of Bangladesh.

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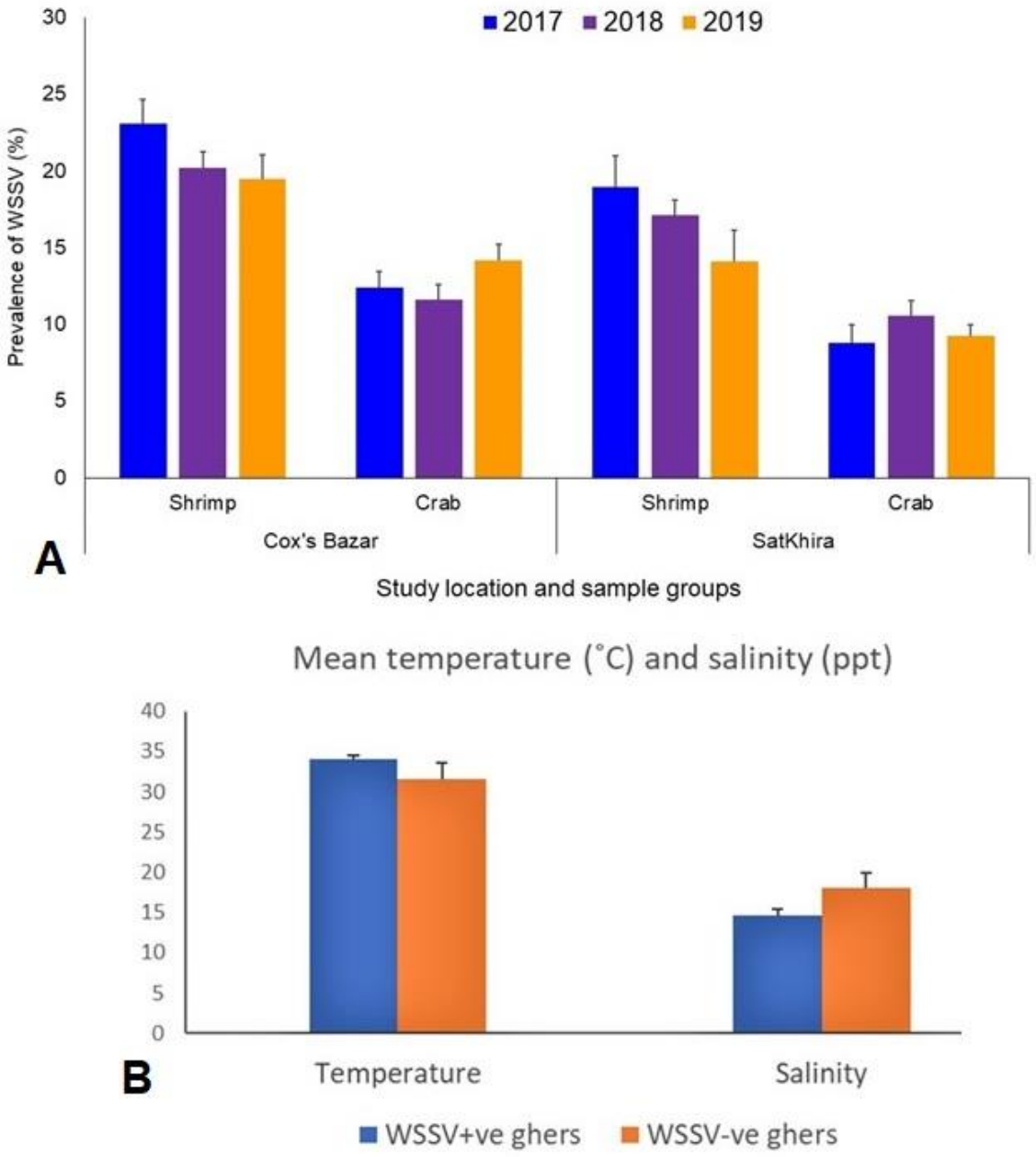

Figure 1. Prevalence of WSSV in Bangladesh. (A). The overall prevalence of WSSV in shrimp and crabs in two different regions (Cox's Bazar and Satkhira) of Bangladesh during 2017-2019. (B) Mean temperature and salinity of shrimp ghers which differed significantly between WSSV +ve and WSSV -Ve ghers.

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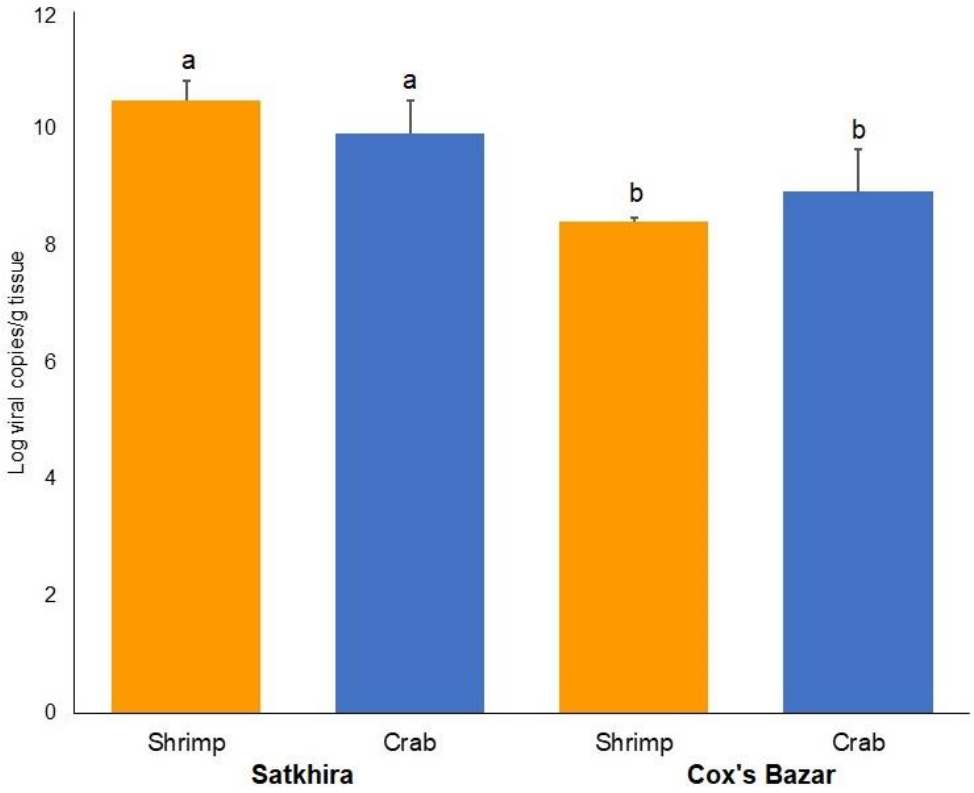

Figure 2. Viral load in crustacean (shrimp and crab) samples in Cox's Bazar and Satkhira.

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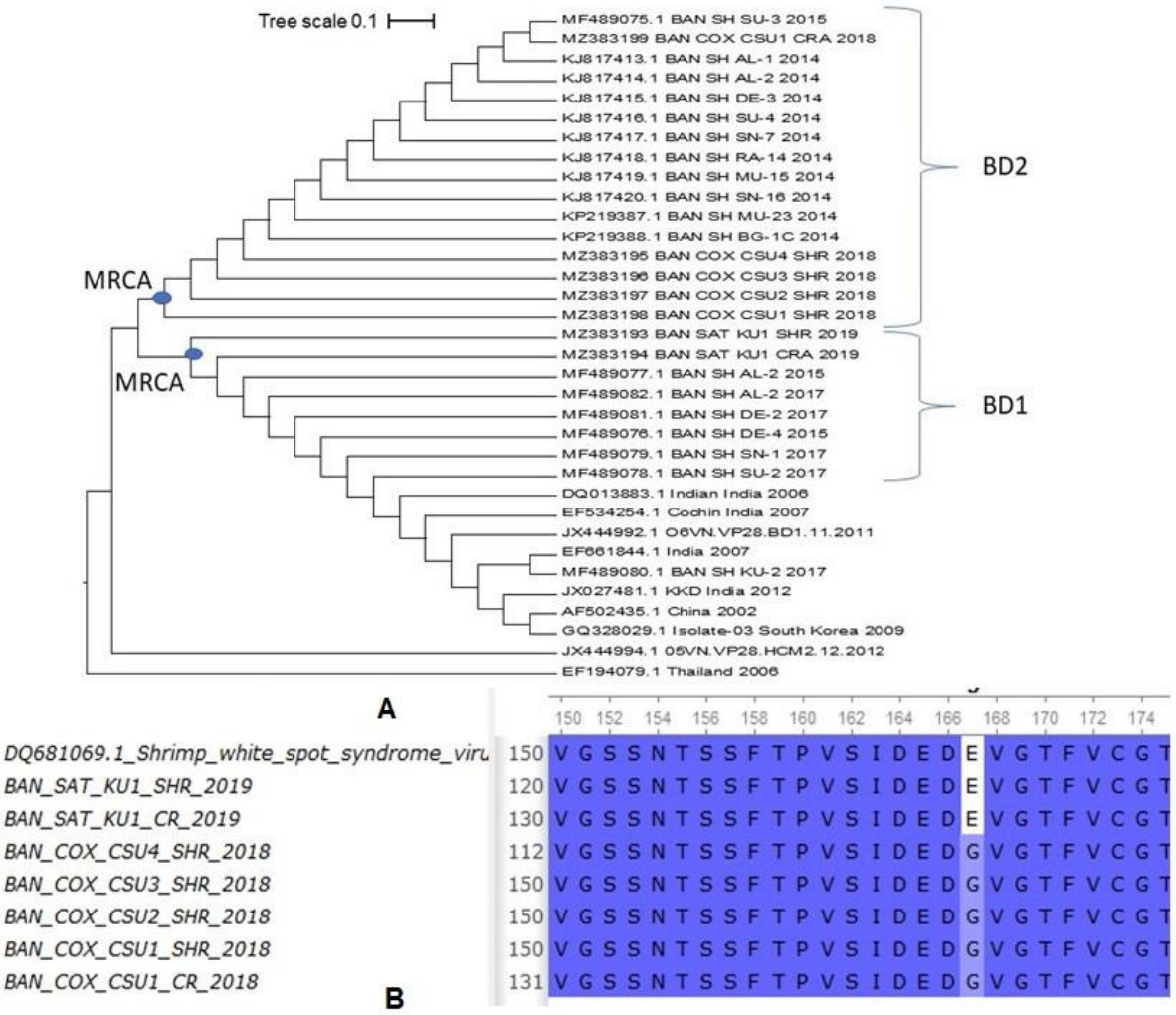

Figure 3. VP28 gene-based phylogenetic analysis. (A) Two phylotypes (BD and BD2) are currently circulating across the country. (B) Amino acid (aa) mutations in the VP28 sequences of WSSV.

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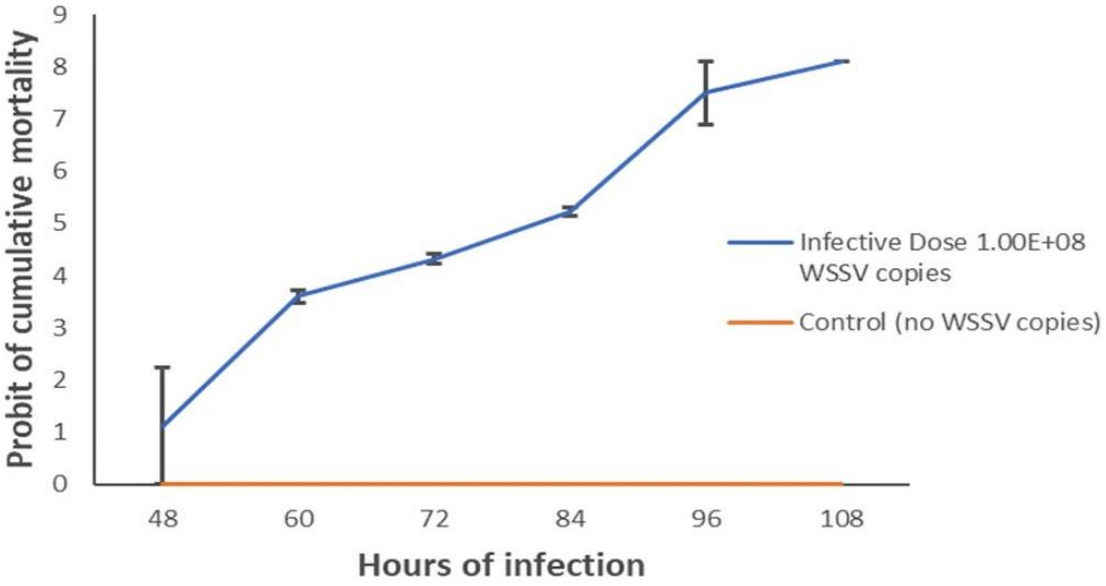

Figure 4. Probit of mortality. The Y-axis represents the morality rates while the X-axis shows the hrs of challenge.

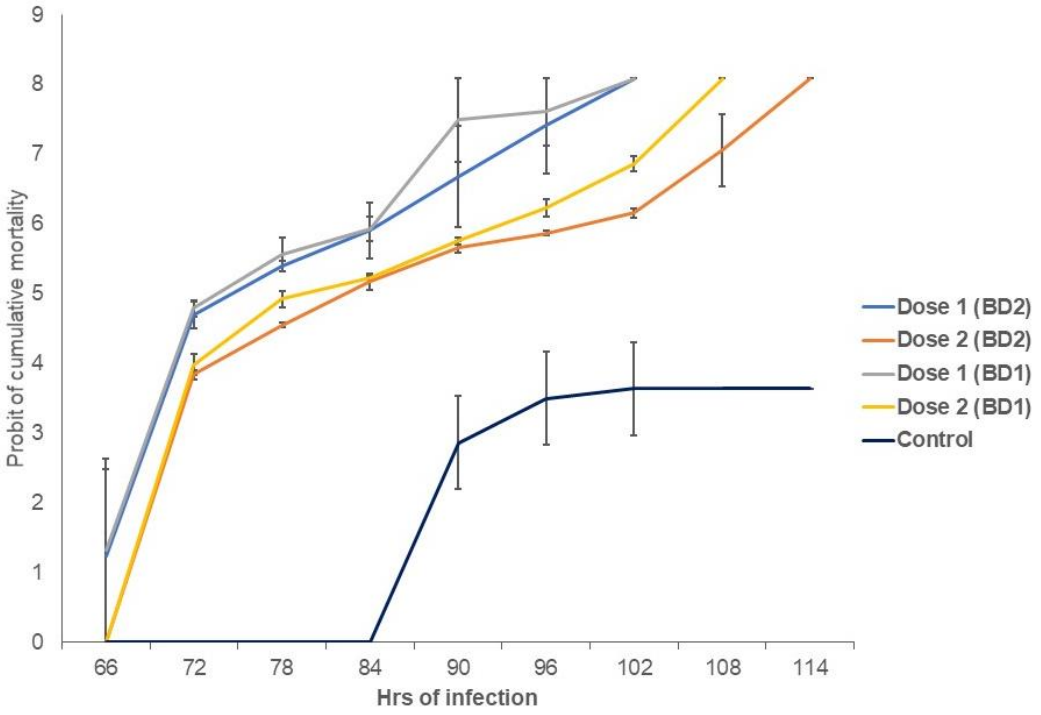

Figure 5. Probit of cumulative mortality after challenge with BD1 and BD2 phylotypes. The Y-axis represents the morality rates while the X-axis shows the hrs of challenge.

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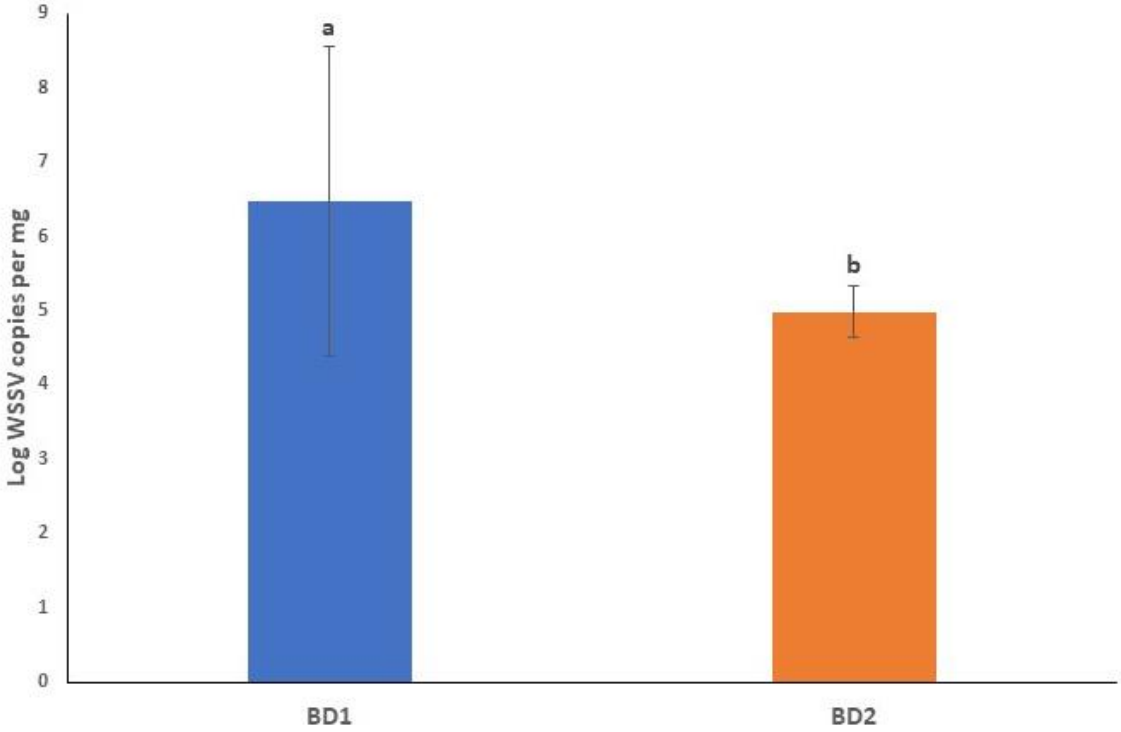

Figure 6. Log WSSV copies per mg of tissue infected with BD1 and BD2. Error bars and 'alphabets' represent the standard deviation and significant difference ($p<0.05$), respectively.

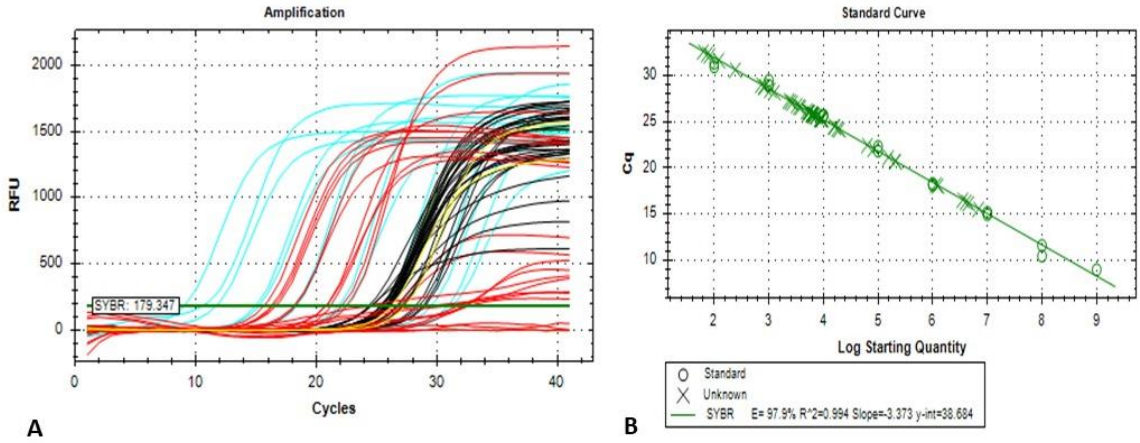

Figure 7. (A) Amplification curves targeting VP28 of WSSV and (B) standard curve produced using quantitative real-time PCR. Plasmid samples with known concentrations were used to obtain the standard curve, and copy numbers of unknown samples were calculated comparing Threshold Cycle values (CT) of samples and standards.

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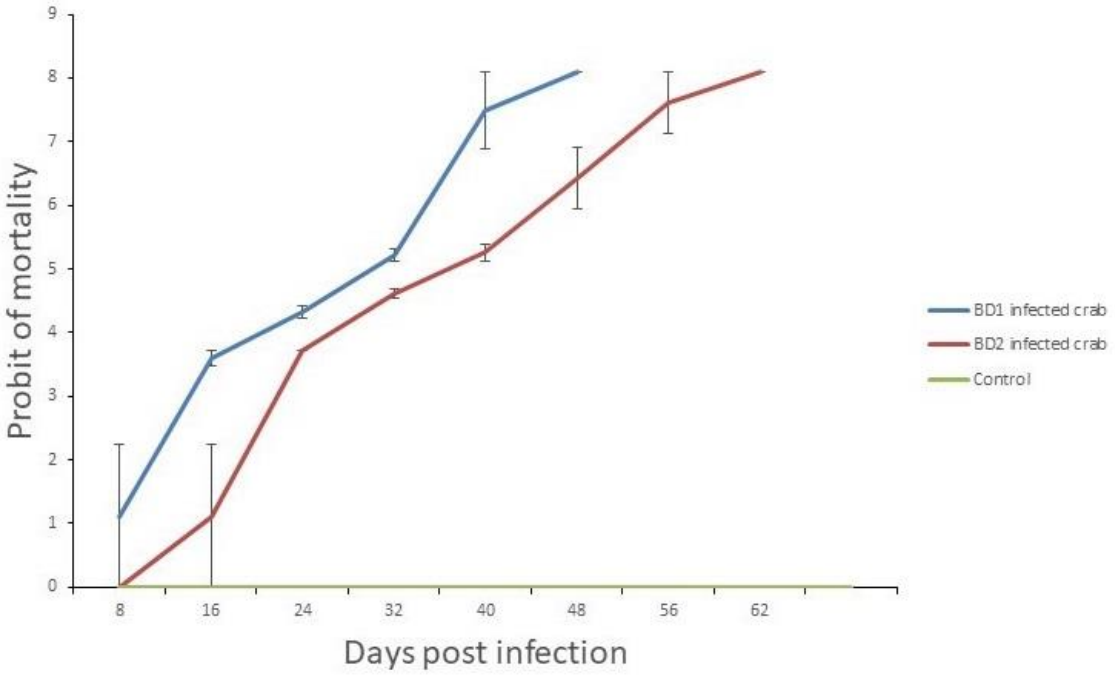

Figure 8. Probit of crab mortality after infection with ingestion method using a dose prepared from BD1 and BD2 infected crab tissue, and control (WSSV-negative crab tissue). The Y-axis represents the morality rates while the X-axis shows the days of post challenge.

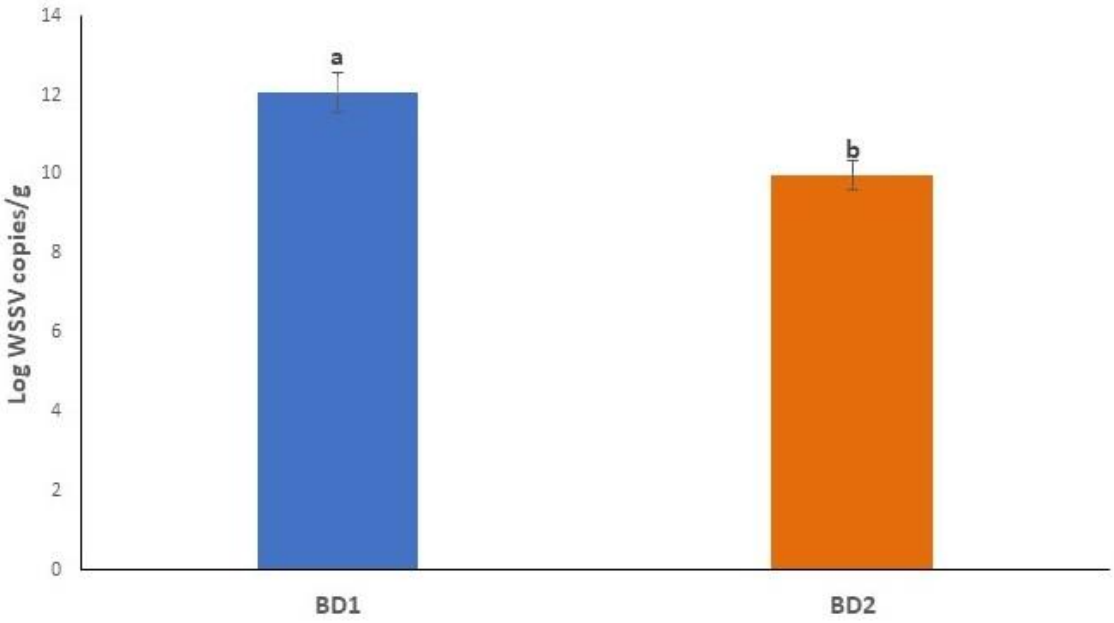

Figure 9. Log WSSV copies per gram tissue of infected crabs of both groups and control. Error bars and 'alphabets' represent standard deviation and significant difference ($p<0.05$), respectively.

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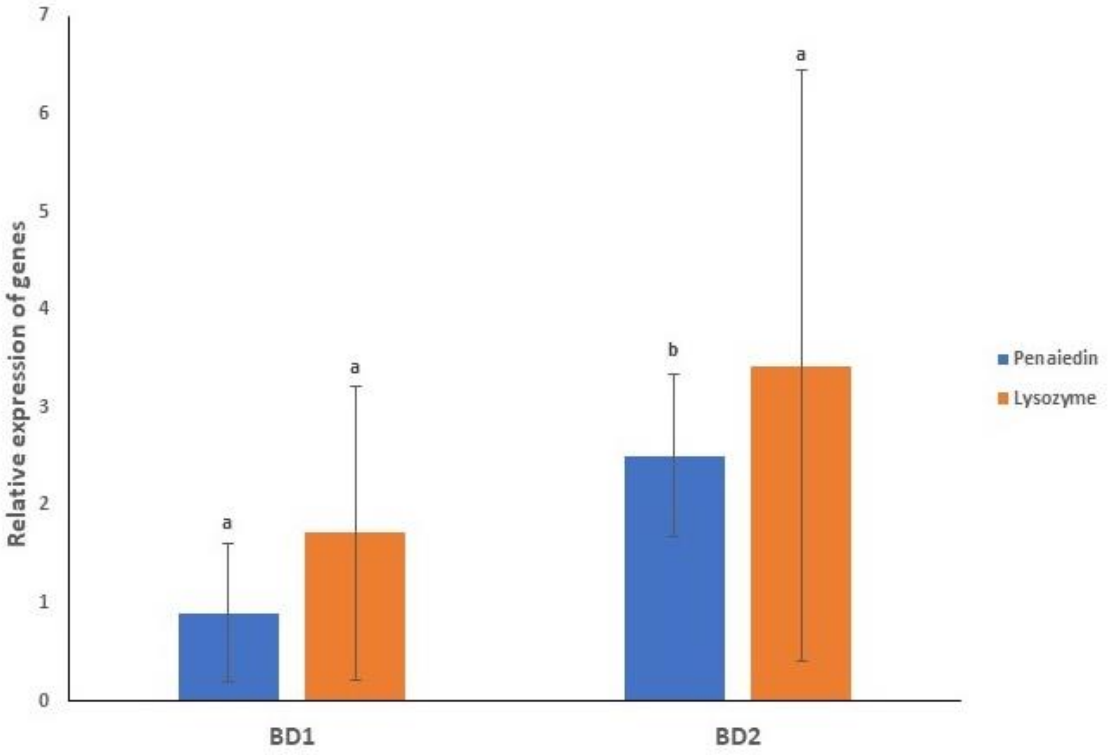

Figure 10. Average relative expression of immunity genes (Penaeidin and lysozyme) in shrimp PL after exposure to WSSV. Error bars and 'alphabets' represent standard error of means and significant differences between the expression of immunity genes, respectively.

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Table 1: 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 districts. In this study, 360 shrimp and 120 crab samples were collected for detection of WSSV using conventional PCR)

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Table 2: C_T values and corresponding WSSV copies from the quantitative real-time PCR (E and G of Sample IDs mean samples challenged with BD1 and BD2)

Sample ID	CT	CT	CT mean	CT SD	WSSV Copies	WSSV copies	Mean WSSV copies/reaction	Mean WSSV copies/mg tissue
G1	27.09	27.21	27.15	0.085	$2.74x10^3$	$2.52x10^3$	$2.63x10^3$	$2.59x10^4$
G2	25.61	25.9	25.755	0.205	$7.53x10^3$	$6.21x10^3$	$6.87x10^3$	$8.41x10^4$
G3	28.80	29.02	28.91	0.156	$8.50x10^2$	$7.35x10^2$	$7.93x10^2$	$1.03x10^4$
G4	25.83	25.77	25.80	0.042	$6.45x10^3$	$6.74x10^3$	$6.60x10^3$	$7.76x10^4$
G5	25.29	25.38	25.34	0.064	$9.37x10^3$	$8.78x10^3$	$9.08x10^3$	$5.56x10^4$
G6	26.05	25.77	25.91	0.198	$5.55x10^3$	$6.72x10^3$	$6.14x10^3$	$6.94x10^4$
G7	25.30	25.35	25.325	0.035	$9.31x10^3$	$8.98x10^3$	$9.15x10^3$	$1.28x10^5$
G8	26.10	26.67	26.385	0.403	$5.37x10^3$	$3.64x10^3$	$4.51x10^3$	$4.16x10^4$
G9	28.21	28.47	28.34	0.184	$1.27x10^3$	$1.07x10^3$	$1.17x10^3$	$1.30x10^4$
G10	25.89	26.05	25.97	0.113	$6.20x10^3$	$5.58x10^3$	$5.89x10^3$	$6.93x10^4$
G11	25.74	24.49	25.12	0.884	$6.87x10^3$	$1.61x10^4$	$1.15x10^3$	$6.89x10^4$
G12	25.85	26.09	25.97	0.17	$6.36x10^3$	$5.40x10^3$	$5.88x10^3$	$7.20x10^4$
G13	25.64	25.86	25.75	0.156	$7.36x10^3$	$6.34x10^3$	$6.85x10^3$	$7.34x10^4$
G14	25.32	25.36	25.34	0.028	$9.16x10^3$	$8.89x10^3$	$9.03x10^3$	$6.85x10^4$
G15	24.30	24.52	24.41	0.156	$1.83x10^4$	$1.58x10^4$	$1.71x10^4$	$2.44x10^5$
G16	25.70	25.46	25.58	0.170	$7.07x10^3$	$8.33x10^3$	$7.67X10^3$	$7.55x10^4$
G17	24.52	24.70	24.61	0.127	$1.58x10^4$	$1.40x10^4$	$1.49X10^4$	$1.82x10^5$
G18	27.10	26.92	27.01	0.127	$2.72x10^3$	$3.07x10^3$	$2.89X10^3$	$3.77x10^4$
G19	27.40	27.04	27.22	0.255	$2.22x10^3$	$2.83x10^3$	$2.5X10^3$	$2.95x10^4$
G20	24.90	24.68	24.79	0.156	$1.22x10^4$	$1.42x10^4$	$1.32X10^4$	$8.06x10^4$
G21	24.27	24.37	24.32	0.071	$1.88x10^4$	$1.75x10^4$	$1.81X10^4$	$2.05x10^5$
G22	23.98	23.48	23.73	0.354	$2.29x10^4$	$3.22x10^4$	$2.71X10^4$	$3.79x10^5$
G23	24.00	24.26	24.13	0.184	$2.26x10^4$	$1.89x10^4$	$2.06X10^4$	$1.91x10^5$
G24	26.53	27.03	26.78	0.354	$4.01x10^3$	$2.85x10^3$	$3.38x10^3$	$3.76x10^4$
G25	23.85	24.35	24.1	0.354	$2.50x10^4$	$1.78x10^4$	$2.10x10^4$	$2.48x10^5$
G26	20.95	21.23	21.09	0.198	$1.81x10^5$	$1.49x10^5$	$1.64x10^5$	$9.87x10^5$
G27	24.63	25.09	24.86	0.325	$1.47x10^4$	$1.07x10^4$	$1.25x10^4$	$1.54x10^5$
G28	23.50	23.88	23.69	0.269	$3.17x10^4$	$2.45x10^4$	$2.79x10^4$	$2.99x10^5$
G29	23.60	24.08	23.84	0.339	$2.96x10^4$	$2.14x10^4$	$2.52x10^4$	$1.91x10^5$
G30	22.22	22.62	22.42	0.283	$7.61x10^4$	$5.79x10^4$	$6.63x10^4$	$9.48x10^5$
E1	33.48	23.65	28.565	6.951	$3.48x10^1$	$4.24x10^4$	$2.12x10^4$	$9.50x10^4$
E2	32.19	32.35	32.27	0.113	$8.44x10^1$	$7.54x10^1$	$7.99x10^1$	$8.88x10^2$
E3	31.15	29.51	30.33	1.16	$1.71x10^2$	$5.24x10^2$	$3.48x10^2$	$1.86x10^3$
E4	12.37	7.65	10.01	3.338	$6.0x10^7$	$1.59x10^9$	$8.25x10^8$	$1.10x10^{10}$
E5	18.23	18.11	18.17	0.085	$1.16x10^6$	$1.25x10^6$	$1.21x10^6$	$9.04x10^6$
E6	30.63	24.20	27.415	4.547	$2.44x10^2$	$1.97x10^4$	$9.97x10^3$	$1.03x10^5$
E7	7.19	28.84	18.015	15.31	$2.17x10^9$	$8.29x10^2$	$1.09x10^9$	$7.15x10^9$
E8	16.38	16.58	16.48	0.141	$4.10x10^6$	$3.56x10^6$	$3.83x10^6$	$2.74x10^7$
E9	26.82	26.59	26.705	0.163	$3.30x10^3$	$3.86x10^3$	$3.58x10^3$	$3.07x10^3$
E10	23.36	23.65	23.505	0.205	$3.48x10^4$	$2.85x10^4$	$3.17x10^4$	$1.62x10^5$
E11	20.76	20.74	20.73	0.014	$2.06x10^5$	$2.08x10^5$	$2.07x10^5$	$5.18x10^5$
E12	22.48	22.08	22.28	0.283	$6.37x10^4$	$8.33x10^4$	$7.35x10^4$	$1.91x10^5$
E13	16.22	15.60	15.91	0.438	$4.57x10^6$	$6.99x10^6$	$5.78x10^6$	$4.08x10^7$
E14	16.92	21.17	19.05	3.005	$3.38x10^7$	$1.56x10^5$	$1.70x10^7$	$7.78x10^7$

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E15	32.60	31.65	32.125	0.672	$6.35x10^{1}$	$1.21x10^{2}$	$9.23x10^{1}$	$3.31x10^{2}$
E16	23.96	23.34	23.65	0.438	$2.32x10^{4}$	$3.54x10^{4}$	$2.87x10^{4}$	$1.28x10^{5}$
E17	12.21	12.53	12.27	0.226	$7.06x10^{7}$	$5.67x10^{7}$	$6.78x10^{7}$	$7.53x10^{8}$
E18	23.23	23.73	23.48	0.354	$3.82x10^{4}$	$2.71x10^{4}$	$3.22x10^{4}$	$1.72x10^{5}$
E19	12.05	12.69	12.37	0.453	$7.87x10^{7}$	$5.09x10^{7}$	$6.33x10^{7}$	$8.44x10^{8}$
E20	15.44	15.20	15.32	0.170	$7.78x10^{6}$	$9.17x10^{6}$	$8.45x10^{6}$	$6.34x10^{7}$
E21	12.00	11.26	11.63	0.523	$8.15x10^{7}$	$1.35x10^{8}$	$1.05x10^{8}$	$1.09x10^{9}$
E22	19.88	19.38	19.63	0.354	$3.75x10^{5}$	$5.29x10^{5}$	$4.46x10^{5}$	$2.94x10^{6}$
E23	12.02	12.42	12.22	0.283	$8.04x10^{7}$	$6.12x10^{7}$	$7.01x10^{7}$	$5.01x10^{8}$
E24	21.95	22.39	22.17	0.311	$9.14x10^{4}$	$6.77x10^{4}$	$7.87x10^{4}$	$6.75x10^{4}$
E25	26.40	26.82	26.61	0.297	$4.38x10^{3}$	$3.29x10^{3}$	$3.80x10^{3}$	$1.95x10^{4}$
E26	16.02	16.86	16.44	0.594	$5.24x10^{6}$	$2.95x10^{6}$	$3.93x10^{6}$	$9.83x10^{6}$
E27	12.25	11.79	12.02	0.325	$6.87x10^{7}$	$9.40x10^{7}$	$8.04x10^{7}$	$2.09x10^{8}$
E28	18.40	16.44	17.42	1.386	$1.03x10^{6}$	$3.93x10^{6}$	$2.01x10^{6}$	$1.42x10^{7}$
E29	17.10	16.74	16.92	0.255	$2.51x10^{6}$	$3.20x10^{6}$	$2.83x10^{6}$	$1.30x10^{7}$
E30	16.70	16.24	16.47	0.325	$3.29x10^{6}$	$4.51x10^{6}$	$3.85x10^{6}$	$1.38x10^{7}$

Authors' contribution

MMH carried out the studies (sampling, laboratory experiments, molecular study and data analysis), and drafted the initial manuscript. MNH critically interpreted the results, produced some of the figures, and edited the manuscript. FA and MIMH helped in sample collection, sample preparation, laboratory experiments and drafting the initial manuscript. MS and MAH developed the hypothesis, supervised the work, and critically reviewed the final manuscript. Finally, all authors read and approved the final manuscript.

Data availability

The reported WSSV sequences of this study have been submitted to the GenBank database under the accession numbers MZ283193 to MZ283199.

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Competing interests

The authors declare no competing interests.

Ethical statement

Not applicable

Supplementary information

Supplementary information supporting the results of the study are available in this article as Figures S1-S6 and Tables S1-S3.

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