SYBR GREEN qPCR BASED QUANTIFICATION OF WSSV BETWEEN SEASONS IN SOUTHWEST BANGLADESH

A thesis submitted to the Department of Fisheries, University of Dhaka in partial fulfilment of the requirements for the degree of Master of Science (MS) in Fisheries

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Abstract

Shrimp aquaculture is a progressive economic growth sub-sector of Bangladesh. The main impediment of shrimp farming is the outbreak of White Spot Disease (WSD) caused by White Spot Syndrome Virus (WSSV) which causes almost 100% mortality within 3-10 days. The prevalence and viral load of WSSV is not similar across all seasons of the year. SYBR green based qPCR provides an effective way for quantification of viral load. Sequencing gives the strain variation of WSSV circulating in Bangladesh. The present study, therefore, was designed to investigate the prevalence and viral load of WSSV in south-west coastalregion in Bangladesh.

Total 48 dead shrimp samples were drawn from four sampling sites such as Satkhirasadar, Tala, Debhata and Assasuniupazillas of Satkhira district. After DNA extraction, the samples were analyzed by VP28 gene specific primers. The SYBR green PCR was performed by using 124bp amplicon for VP28 gene. The results were analyzed by SPSS and Microsoft Excel. The computational bioinformatics tools were used to perform molecular analysis.

Conventional PCR showed that the prevalence in three seasons (Immediate Before Monsoon: 75%; During Monsoon: 81.25% and Immediately After Monsoon: 93.15%) had relationship with the viral load $3.52 \pm 0.22 \times 10^8$ copy/g of tissue, $5.43 \pm 0.25 \times 10^9$ copy/g of tissue $8.63 \pm 0.23 \times 10^{11}$copy/g of tissue, respectively. Protein BLAST of sequenced WSSV positive amplicon of VP28 amplicon (BAN_323_S3_2015_VP28) showed a mutation with NCBI reference sequences. Phylogenetic analysis of WSSV positive amplicon of VP28 indicates that WSSV Bangladeshi strain is phylogenetically related to India.

WSSV infections are widely distributed in Satkhira and towards the coast the prevalence and viral load could be increased. Therefore, proper farm management is required to improve yield of shrimp.

This study has indicated that SYBR Green qPCR technique can be successfully applied in detecting WSSV load 1000 times sensitive than conventional PCR method. WSSV load can vary between seasons and within sampling sites. WSSV prevalence had similar trend. Discovery of a new mutation in the VP28 gene denotes genetic persistent change in the genome.

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ABBREVIATIONS

BLAST	Basic Local alignment Search Tool
DDBJ	DNA Data Bank of Japan
ElISA	Enzyme linked Immuno Sorbent Assay
WSD	White Spot Disease
WSSV	White Spot Syndrome Virus
MEGA	Molecular Evolutionary Genetics Analysis
MSA	Multiple Sequence Alignment
NCBI	National Centre for Biotechnology Information
NTP	Nucleotide Phosphate
PDB	Protein Data Bank
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
IBM	Immediate Before Monsoon
DM	During Monsoon
IAM	Immediate After Monsoon

ABBREVIATED NAME 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 Aspartate

E Glutamate

K Lysine

R Arginine

C Cysteine

H Histidine

Dedicated to

My parents who cherished my life with their blessings

Certification

We certify that this thesis entitled "**SYBR Green qPCR based Quantification of WSSV between Seasons in Southwest Bangladesh"** is submitted by**Md. Inja-Mamun Haque**, Roll No.: Curzon-711, Session: 2014-15, Registration No. 2010-912-988/2010-11, has been carried out under our supervision.

This is further to certify that it is an original work and suitable in partial fulfillment of the requirementsfor the degree of Master of Science (MS) in Fisheries from University of Dhaka.

We wish every success in his life.

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Introduction

Chapter 1

1.1 Background

Crustacean, forming a notably large group of arthropods, are consumed by humans worldwide due to their delicious taste and high nutritional value (Chakrabarty*et al*., 2014). Among the various lineages of crustacean, shrimp (*Penaeusmonodon*) is one of the most lucrative species (second highest foreign currency earning source) in Bangladesh. Shrimp aquaculture plays a vital role for the socio-economic development of Bangladesh as it generates a prosperous sustainable job for rural and urban community (Hossain *et al*., 2014). Most (97%) of the shrimp produced are exported which contribution is about 5% to the national GDP (Rahman and Hossain, 2013). Nearly 8.5 million Bangladeshi people, particularly coastal regions peoples, directly depends on this sector for their livelihood (DoF, 2013). However, this industry has suffered from many diseases (virus, bacteria, protozoa, etc.) among which various viral diseases are the leading ones. More than twenty viral pathogens reported to infect shrimps, whereas, among them, nine are liable for significant losses due to huge mortality, counting white spot syndrome virus (WSSV) as the deadliest (Lightner*et al*., 1998). WSSV (gross clinical signs of this disease is the appearance of white spots at the cephalothorax region of shrimp cuticle) causes white spot disease (WSD) which was first reported in 1992 in kurma prawn (*Penaeus japonicas*) in northern Taiwan and the syndrome had spread by importing shrimp to southern Japan, through Thailand, into Indonesia, west India and as far as the coast of Bangladesh (CSIRO, 2002). WSSV is a highly lethal, stress-dependent, rod-shaped dsDNA virus, classified into the genus Whispovirus of the family Nimaviridae (Yang *et al*., 2001), capable of transmitting both vertically from infected mother shrimp to post-larvae and horizontallythrough water or cannibalism of moribund shrimp (Kanchanaphum*et al*., 1998; Sanchez-Paz, 2010).

1.2 An overview of WSSV, Conventional PCR, Real-Time PCR and Bioinformatics

1.2.1 Overview of WSSV

1.2.1.1 History of WSSV in Shrimp Aquaculture Industry

One of the biggest threat to the shrimp aquaculture industry is viral disease. Prior to 1991, the global shrimp production had been increasing consistently year after year since its data was first recorded in 1970. For the first time, in that year, a disease known as

White Spot Syndrome began to cause devastating fatalities in the Taipei Taiwanese province's shrimp farms (Escobedo-Bonilla *et al*., 2008). The WSSV outbreak chronology (**Table 1.1)** indicates that within ten years, from the first outbreak, it circulates across the world.

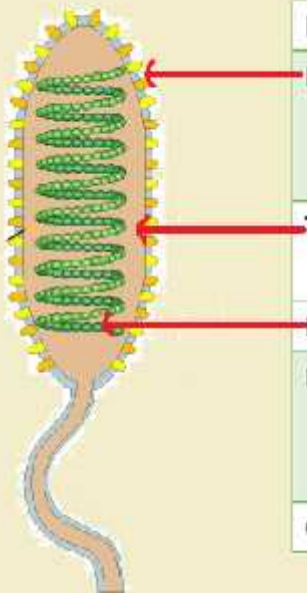
Table 1.1 Chronology of white spot syndrome virus outbreaks in shrimp farming countries in Asia and America (Escobedo-Bonilla *et al*., 2008)

Year first reported	Country	References
1992	Taiwan	Chou *et al*., 1995
1993	China, Japan, Korea	Zhan *et al*., 1998; Inouye *et al*., 1994; Park *et al*., 1998
1994	Thailand, India, Bangladesh	Lo *et al*., 1996; Karunasagar*et al*., 1997; Mazid andBanu, 2002
1995	USA	Lightner, 1996; Wang *et al*., 1999a
1996	Indonesia, Malaysia, Sri Lanka	Durand *et al*., 1996; Kasornchandra*et al*., 1998; Rajan*et al*., 2000
1997	Vietnam	Bondad-Reantaso*et al*., 2001
1998	Peru	Rosenberry, 2001
1999	Philippines, Ecuador, Colombia	Magbanua*et al*., 2000; Bondad-Reantaso*et al*., 2001; Panama *et al*., 2001; Wu *et al*., 2001
1999-2000	Mexico	Bondad-Reantaso*et al*., 2001
2002	France, Iran	Dieu*et al*., 2004; Marks, 2005
2005	Brazil	APHIS-USDA, 2005

1.2.1.2 Genomics and Proteomics of WSSV

The genomes of three WSSV isolates from different geographical locations have been sequenced completely, which includes, a) 292.9 kb isolated from Thailand (WSSV-TH) (AF369029), b) 307.2 kb isolated from Taiwan (WSSV TW) (AF440570) and c) 305.1 kb isolate from China AF332093 (WSSV-CN) (Chen *et al*.,2002; Yang *et al*., 2001).

There are mainly four types of protein (**Figure 1.1)** found in WSSV that binds with shrimp binding protein.



Protein Type	WSSV Protein	Shrimp Binding Protein
Envelop protein	VP28	PmRab7, FcLec3, Hsc70, LvCTL1
	VP187	ß-integrin
	VP664	PjRab
Tegument	VP26	ß-Actin, WBP, LvCTL1
	VP24	LvCTL1
Nucleocapsid	VP15	PmFKBP46
Non-structural	VP9	Pm-RACK1
	WSSV427,403	PPs
	WSSV227, 304	FcUbc
Others	Purified virion	hemocyanin

Figure 1.1 Schematic diagram of WSSV proteins that interact with shrimp binding proteins (Leu*et al*., 2009)

VP28 is the major envelope protein that has an important role in infection (Xie*et al*., 2006). The presence of multiple glycosylation sites of VP28 is gathered to contribute in the recognition of receptors from the shrimp cell surface (Yi *et al*., 2004) and adopt ß-barrels architecture with a protruding region (Tang *et al*., 2007).

1.2.1.3 Host Range and Transmission of WSSV

To date, more than 93 species of arthropods (Lo *et al*., 1998), including salt, brackish and freshwater penaeids(*Penaeusmonodon*, *Marsupenaeusjaponicus*, *Fenneropenaeuschinensis*, *Macrobrachiumrosenbergii*and *Procambarus clarkia*), crabs and crayfish (Fauquet, 2005), have been reported as hosts or carriers of WSSV. Many species, rotifer, phytoplankton, marine molluscs, polychaete worms (Vijayan*et al*., 2005) and non-decapod crustaceans including *Artemiasalina*(Chang *et al*., 2002) and the copepods, as well as non-crustacean aquatic arthropods and Euphydradae insect larvae, are not necessarily the natural hosts of WSSV, but may only act as vectors(Lo *et al*.,1998).

1.2.1.4 Target Organ, Tissues and Mechanism of Spread of WSSV infection

The major targets for WSSV infection**(Figure 1.2)** are tissues of ectodermal and mesodermal origin(Rajendran*et al*., 2001) including those of the epidermis, gills, foregut, hindgut, antennal gland, lymphoid organ, gonads, haematopoietic cells and cells associated with the nervous system (Reddy *et al*,. 2010). Epithelial cells of organs of endodermal origin such as the hepatopancrease, anterior and posterior midgut caeca and midgut trunk are refractory to WSSV infection (Hameed *et al*., 1998). The primary sites of WSSV replication in early juvenile *Penaeusmonodon*were found out as subcuticular epithelial cells of the stomach and cells in the gills, in the integument and in connective tissue of the hepatopancrease determined by *in situ* hybridization (Chang *et al*., 1996). One opinion is that, the virus circulates through the hemolymph to the target organs (Di Leonardo *et al*., 2005)

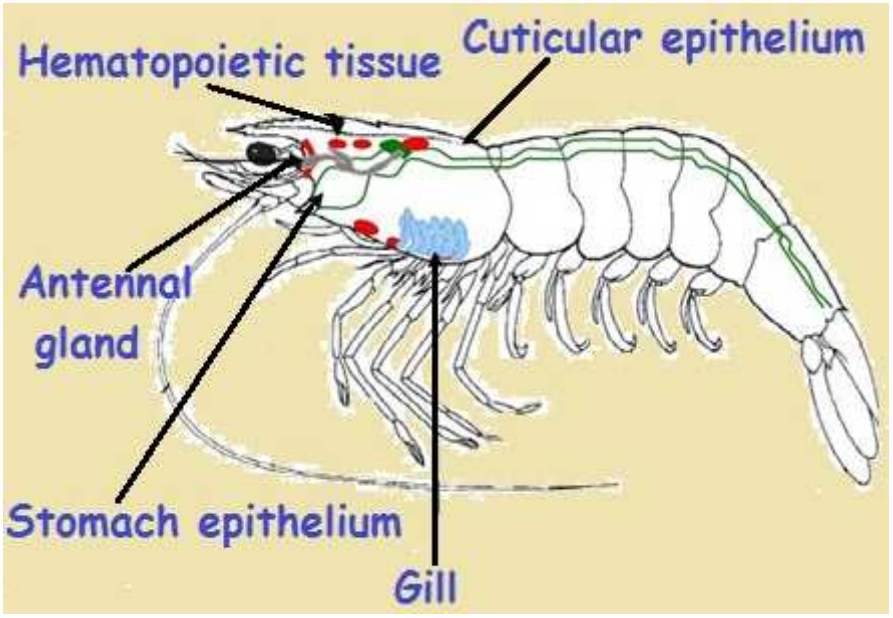


Figure 1.2 Major target tissues of WSSV replication in shrimp (Rahman *et al*., 2006)

1.2.1.5 Clinical Sign and Pathology

WSSV infected shrimp display clinical signs such as anorexia, lethargia, swollen branchiostegites due to fluid accumulation, white spots in the cuticle, separated/ loose cuticle from underlying epidermis, yellowish-white and enlarged hepatopancrease, hemolymph which fails to coagulate and reddish discoloration of the moribund shrimp

(Shapiro-lan*et al*., 2005; Perez*et al*., 2005; Marks, 2005;Wang*et al*., 1999). Clinical signs do not allow a diagnosis of WSSV (Flegel, 2006) because anorexia is observed in uninfected shrimp before and after molting, white spots in the carapace**(Figure 1.3)** can also be caused by bacterial infection (Wang*et al*., 2000).

Figure 1.3 Gross signs of WSSV infection. A) White inclusions within the cuticle (arrows) of moribund shrimp cannot be scraped-off with the thumbnail B) Tissue after severe WSSV infection (Flegel, 2006).

The outbreak of WSSV is mainly three types **(Table 1.2)**which causes significant mortality within a few days

Table 1.2 WSSV outbreaks in penaeid shrimps based on the clinical manifestation (Sudha*et al*., 1998)

Outbreak Type	Tissue level severity	Severity of Infection	Mortalities
Type I	moderate to high	affected shrimp had prominent white spots on the carapace, as the principal clinical sign	significant mortalities occurred within 7-10 days
Type II	very high	affected shrimp displayed massive reddening, the tissue level severity of the infection was very high	mass mortalities occurred within 2-3 days
Type III	low	white spots and reddening were absent	mortalities of shrimp were spread over a duration of

	15-28 days.

1.2.1.6 Molecular Basis of Viral Host Interaction

The viral infection starts with the attachment of the virions onto the cell surface receptors (Sritunyalucksana*et al*., 2006). Two of the major WSSV envelope proteins known to be involved in the interaction with host cells are VP28 and VP19 (Escobedo-Bonilla*etal.,* 2008). Asmore becomes known about the structure of the WSSV virion, it is becoming clear that the many structural proteins are interacting with each other, forming protein complexes in the envelope (Chang *et al.*, 2010; Li *et al*., 2006;Leu*et al.*, 2005).At the cellular level, a shrimp protein called *Penaeusmonodon*Rab7 (PmRab7),identified from the membrane of hemocytes, may function as one of the receptors forthe virus (Sritunyalucksana*et al.,* 2006). It binds directly to the major viral envelopeprotein VP28 and is present in most shrimp tissues (Flegel, 2007).*In vivo* neutralization assaysdemonstrated that PmRab7 is essential for infection. Other researchers haveconcluded that the Rab-dependent signaling complex might act as a virus recognitionprotein that triggers a phagocytic defense against the virus, which aids in fightinginfection (Wu *et al.,* 2007).

1.2.1.7 Methods for Detection and Quantification of WSSV in Shrimps

The traditional procedure for disease diagnosis greatly depended upon the culture of pathogenic microbes, analysis of phenotypic or serological properties of the pathogen and histological studies of the affected host tissue.The modern molecular biology presents the opportunity to detect the pathogen directly, targeting the genetic material replacing culture, serological or histological techniques (Liu *et al*., 2002; Kiatpathomchai*et al*., 2001; Otta*et al*., 1999; Lightner and Redman, 1998).

The selection of a method is dependent on the purpose. The PCR technique is the best recommended method**(Table 1.3)** by OIE (Office Des Epizooties). The PCR technique include conventional PCR, nested PCR and Real-Time PCR. In Real-Time PCR detection as well as quantification can performed simultaneously.

Table 1.3. Description of each diagnostic method for screening of WSSV according to OIE (Claydon*et al.*, 2004)

Method	Surveillance				Disease diagnosis	
	Larvae	PLs	Juvenile	Adults	Presumptive	Confirmatory
Gross signs	D	C	C	D	C	D
Bioassays	D	C	D	D	C	C
WML microscopy	D	C	C	D	C	C
Histopathology	D	C	C	C	B	B
TEM	D	D	D	D	D	A
Ig- based Methods	D	C	D	D	B	B
DNA-probes	C	B	B	C	A	A
PCR	A	A	A	A	A	A
Sequence	D	D	D	A	D	A
From A: most suitable method to D: not recommended method						

1.2.2 Overview of Conventional PCR

PCR has completely revolutionized the detection of RNA and DNA. Undoubtedly PCR**(Figure 1.7)**has been established, since its invention in the mid-1980s by Kary Mullis, as the most important diagnostic technique in clinical and pathological microbiology.

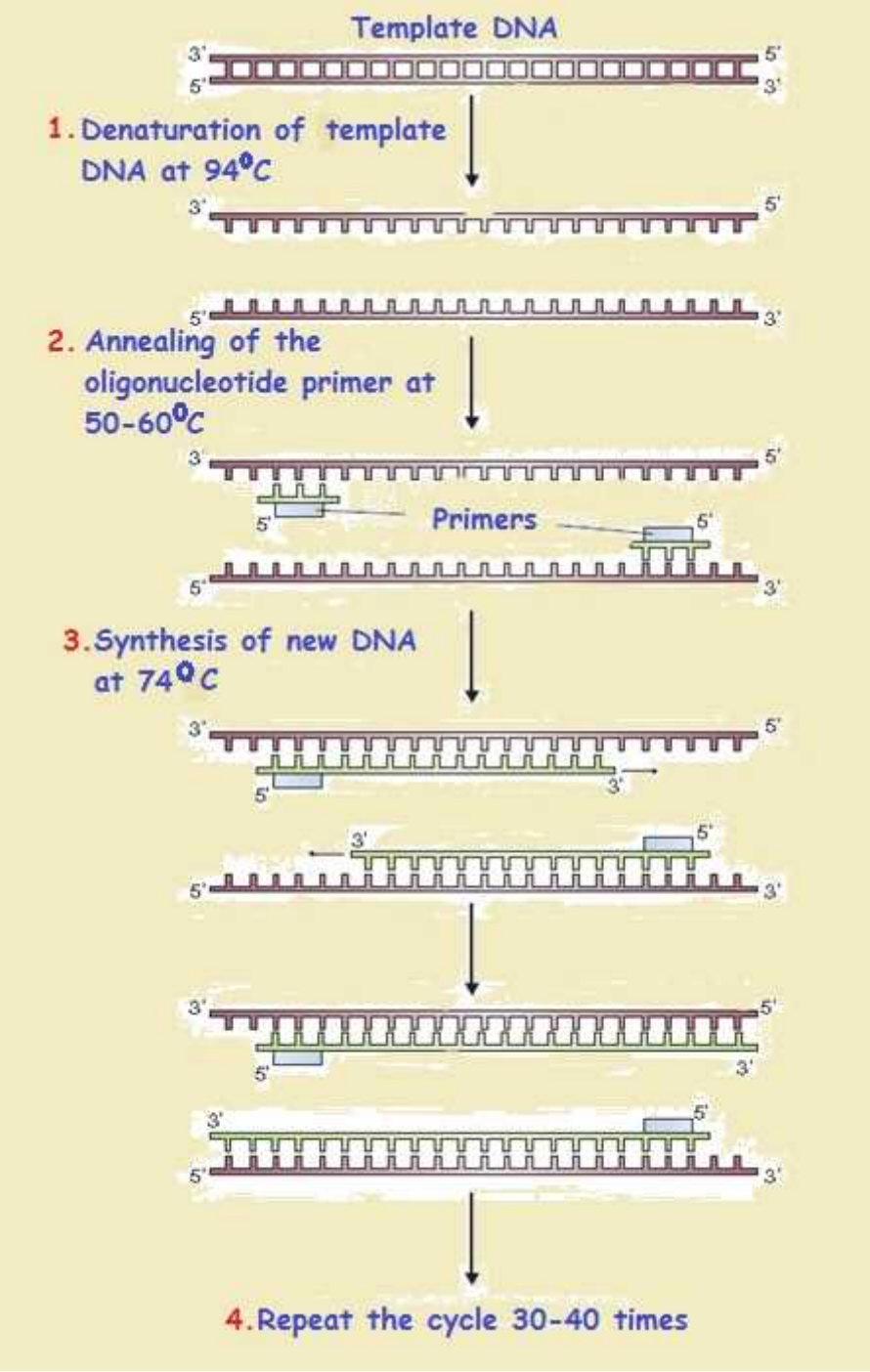


Figure 1.4: Polymerase chain reaction (Brown, 2010)

1.2.3 Overview of Real-Time PCR

1.2.3.1 Basic Principle of Real-Time PCR Based Detection

Real-time PCR is a variation of the standard PCR technique used to quantify DNA or RNA in a sample. Using sequence-specific primers, the relative number of copies of a

particular DNA or RNA sequence can be determined. By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. If a particular sequence (DNA or RNA) is abundant in the sample, amplification is observed in earlier cycles; if the sequence is scarce, amplification is observed in later cycles. Quantification of amplified product is obtained using fluorescent probes or fluorescent DNA binding dyes and real-time PCR instruments that measure fluorescence while performing temperature changes needed for the PCR cycles (Bustin, 2000).The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescent dye scanning capability. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction (Biosystem, 2008).

1.2.3.2 Advantage of Real-Time PCR

Real time PCR is more advantageous than conventional PCR for some which are described below:

1. Real-Time PCR allows detection of low copy targets (high sensitivity)
2. Ability to precisely measure the amount of amplicon at each cycle, which allows highly accurate quantification of the amount of starting material in samples.
3. Amplification and detection occurs in a single tube, eliminating post-PCR manipulations
4. Real time quantification of target amplification as the amplicon copy number increases, fluorescence increases.
5. Real time PCR is quick and it saves time.

1.2.3.3 Real-Time PCR Steps

There are three major steps that make up each cycle in a real-time PCR reaction. Reactions are generally run for 40 cycles.

Denaturation

High temperature incubation is used to "melt" double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if template GC content is high.

Annealing

During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (5°C below the Tm of the primer).

Extension

At 70-72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60°C as the temperature.

1.2.3.4 Primer Design

Good primer design is one of the most important parameters in real-time PCR. In general primers should be 18–24 nucleotides in length and the amplicon length should be approximately 50–150 bp, since longer products do not amplify as efficiently. This provides for practical annealing temperatures. Primers should be designed according to standard PCR guidelines (Primer design software programs, such as OligoPerfect™ designer and Primer ExpressR software and sequence analysis software, such as Vector NTIR Software can automatically evaluate a target sequence and design primers for it based on the criteria listed above).

They should be specific for the target sequence and be free of internal secondary structure. Primers should avoid stretches of homopolymer sequences (e.g., poly dG or repeating motifs, as these can hybridize inappropriately. Primer pairs should have compatible melting temperatures (within 5°C) and contain approximately 50% GC content. Primers with high GC content can form stable imperfect hybrids. Conversely, high AT content depresses the Tm of perfectly matched hybrids. If possible, the 3´end of the primer should be GC rich (GC clamp) to enhance annealing of the end that will be extended. Analyze primer pair sequences to avoid complementarity and hybridization between primers (primer-dimers).

1.2.3.5 Real-Time PCR Analysis Terminology

This section provides an overview of the major terms involved in real-time PCR experiments.

Table 1.4 Description of different terms involved in real-time PCR experiments (Biosystem, 2008)

Term	Description
Baseline	The initial cycles of PCR, usually cycles 3 to 15, in which there is little change in fluorescent signal.
Threshold	The level of signal that reflects a statistically significant increase over the calculated baseline signal, automatically determined, used for C_T determination in real-time assays. Usually, RT-PCR software automatically sets the threshold at 10 times the standard deviation of fluorescence value of the baseline. The threshold is the line whose intersection with the Amplification plot defines C_T**(Figure 1.5)**.
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence signal passes the threshold. The C_T is used to calculate the initial DNA copy number, because the C_T value is inversely related to the amount of starting template**(Figure 1.5)**.
Normalized reporter (Rn)	The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.
Delta Rn (ΔRn)	The magnitude of the signal generated by the specified set of PCR

	conditions. (ΔRn = Rn − baseline)
Standard	A sample of known quantity used to construct a standard curve.
Correlation Coefficient (R^2 Value)	A Measure of how well the data fit the standard curve. The R^2value reflects linearity of the standard curve. Ideally, $R^2 = 1$, although 0.999 is generally the maximum value.
Slope	The slope of the log–linear phase of the amplification reaction is a measure of reaction efficiency. To obtain accurate and reproducible results, reactions should have efficiency as close to 100% as possible, equivalent to a slope of –3.32.
Standard Curve	A dilution series of known template concentrations can be used to establish a standard curve for determining the initial starting amount of the target template or for assessing the reaction efficiency. From this standard curve, information about the performance of the reaction as well as various reaction parameters (including slope, y-intercept, and correlation coefficient) can be derived. The concentrations chosen for the standard curve should encompass the expected concentration range of the target **(Figure 1.6)**.
Y-Intercept	The y-intercept corresponds to the theoretical limit of detection of the reaction, or the C_T value expected if the lowest copy number of target molecules denoted on the x-axis gave rise to statistically significant amplification.
Exponential Phase	At the beginning of the exponential phase, all reagents are still in excess, the DNA polymerase is still highly efficient, and the product, which is present in a low amount, will not compete with the primers'annealing capabilities. All of these things contribute to more accurate data.
Efficiency	Ideally, the efficiency (E) of a PCR reaction should be 100%

	determined by the equation Efficiency = $10^{(-1/slope)}$ -1, meaning the template doubles after each cycle during exponential amplification. A good reaction should have an efficiency between 90% and 110%, which corresponds to a slope of between –3.58 and –3.10.
Dynamic range	This is the range over which an increase in starting material concentration results in a corresponding increase in amplification product. Ideally, the dynamic range for real-time PCR should be 7-8 orders of magnitude for plasmid DNA and at least a 3–4 log range for cDNA or genomic DNA.
Precision	The standard deviation (square root of the variance) is the most common measure of precision. If many data points are close to the mean, the standard deviation is small; if many data points are far from the mean, the standard deviation is large.
Sensitivity	Any system capable of effectively amplifying and detecting one copy of starting template has achieved the ultimate level of sensitivity, regardless of the absolute value of the C_T.
Absolute quantification	Absolute quantification describes a real-time PCR experiment in which samples of known quantity are serially diluted and then amplified to generate a standard curve. Unknown samples are then quantified by comparison with this curve.
Relative quantification	Relative quantification describes a real-time PCR experiment in which the expression of a gene of interest in one sample is compared to expression of the same gene in another sample. The results are expressed as fold change (increase or decrease) in expression of the treated in relation to the untreated.
Nucleic acid target (template)	Nucleotide sequence that wants to detect and quantitate.

No template control (NTC)	A sample that does not contain template. It is used to verify amplification quality.
Unknown sample	A sample containing an unknown quantity of template that need to characterize.

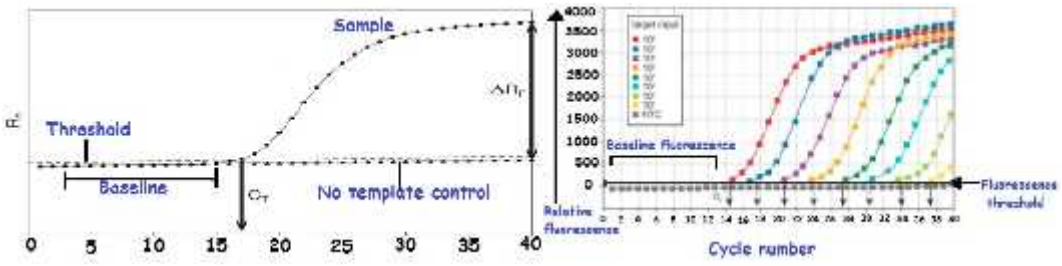


Figure1.5 A representative amplification plot (Relative fluorescence vs. cycle number)

Amplification plots are created when the fluorescent signal from each sample is plotted against cycle number; therefore, amplification plots represent the accumulation of product over the duration of the real-time PCR experiment. The samples used to create the plots are a dilution series of the target DNA sequence.

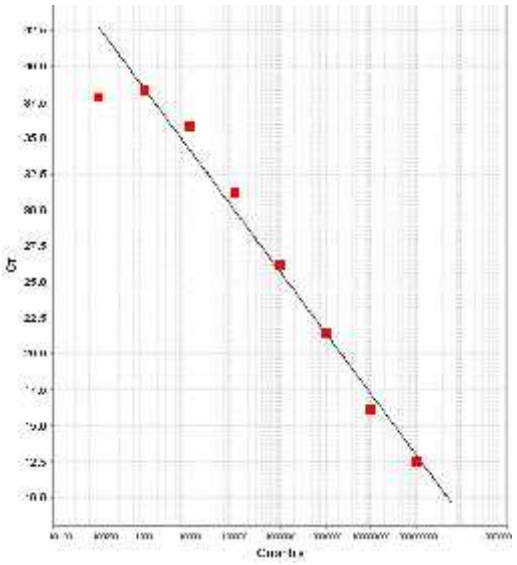


Figure 1.6 Representative standard curve of Real-Time PCR

1.2.3.6 Absolute Quantification

Absolute quantification describes a qPCR experiment in which samples of known quantity are serially diluted and then amplified to generate a standard curve. An unknown sample can then be quantified based on this curve. Standard curve generated in absolute quantification real time assay resemble equation of straight line. Equation of straight line can be presented as, $y = mx + c$

If we correlate the standard curve of straight line with the standard curve generated by Real Time PCR assay, the equation will be as follows:

$C_T = m (\log Quantity) + c$

Or, $m (\log Quantity) = C_T - c$

Or, $\log Quantity = (C_T - c)/m$

Or, $Quantity = 10^{(C_T - c)/m}$

So, $Quantity = 10^{[(C_T - c)/ slope}$

Here,

C_T = Cycle Threshold

m = slope of Standard curve

c = Y- intercept

Standard curve of real time PCR assay is decreasing Straight line which has negative slope.

1.2.3.7 Melting curve and Primer-Dimers

A melting curve charts the change in fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates, or "melts" into single-stranded DNA (ssDNA) as the temperature of the reaction is raised. The specificity of the qPCR or qRT-PCR reaction can be confirmed using melting curve analysis. Primer-dimers occur when two PCR primers with homologous sequences (either same sense primers or sense and antisense primers) bind to each other instead of to the template. Melting curve analysis can identify the presence of primer-dimers because they exhibit a lower melting temperature than the amplicon**(Figure 1.7)**.

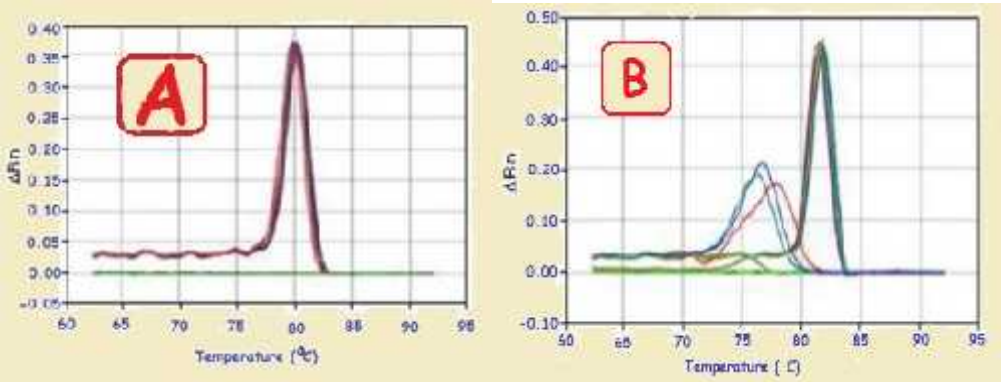


Figure 1.7 Melt curve showing (A) specific product (Single peak) and (B) non-specific product (Multiple peak).

1.2.3.8 SYBR green Based Real-Time PCR Fluorescence Detection Systems

Several different fluorescence detection technologies can be used for real time PCR, and each has specific assay design requirements.SYBR® Green dye will bind to any amplified product, target or non-target, and all such signals are summed, producing a single amplification plot. SYBR® Green amplification plot shape cannot be used to assess specificity. Plots usually have the same appearance, whether the amplification consists of target, non-target, or a mixture. The fact that a SYBR® Green assay produced an amplification should not be automatically taken to mean the majority of any of the signal is derived from target.

Accurate identification of the target peak depends onamplification of pure target. By starting withpure target, the researcher will be able to associate a peakT_M and shape with the particular target after amplification.Only one peak should be observed. The presumptive targetpeak should be narrow, symmetrical, and devoid of otheranomalies, such as shoulders, humps, or splits. Theseanomalies are strong indications that multiple productsof similar T_M's will be produced, casting strong doubts aboutthe specificity of those reactions. Therefore, one, narrow symmetric peak should not be assumed to be the target, nor oneproduct, without additional supporting information.

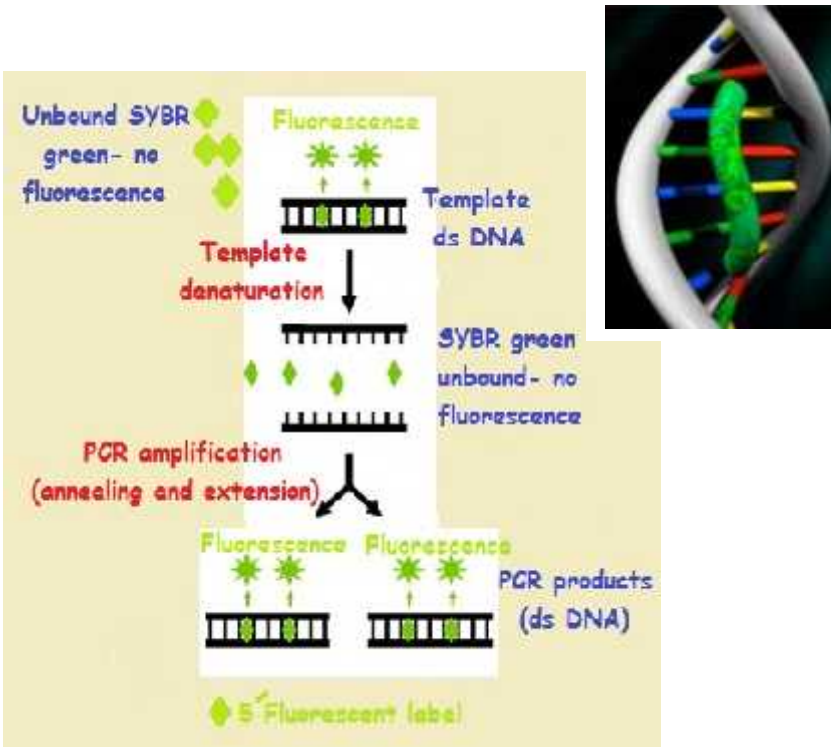


Figure 1.8 Mode of action of SYBR Green dye. Inset SYBR Green binding with DNA (Smith and Osborn,2009)

1.2.3.9 Assay Design

Recombinant plasmid DNA based standard curve the 643 specific amplicon of VP28 is cloned into TA cloning vector. The TA vector is linearized and only PCR purified product is necessary for cloning reaction. *Taq*polymerase has a non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. Topoisomerase I from *Vaccinia*virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand. The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase. TOPO® TA Cloning exploits this reaction to efficiently clone PCR products. The basic mechanism of the TOPO TA cloning reaction is delineated in the **Figure 1.9** below.

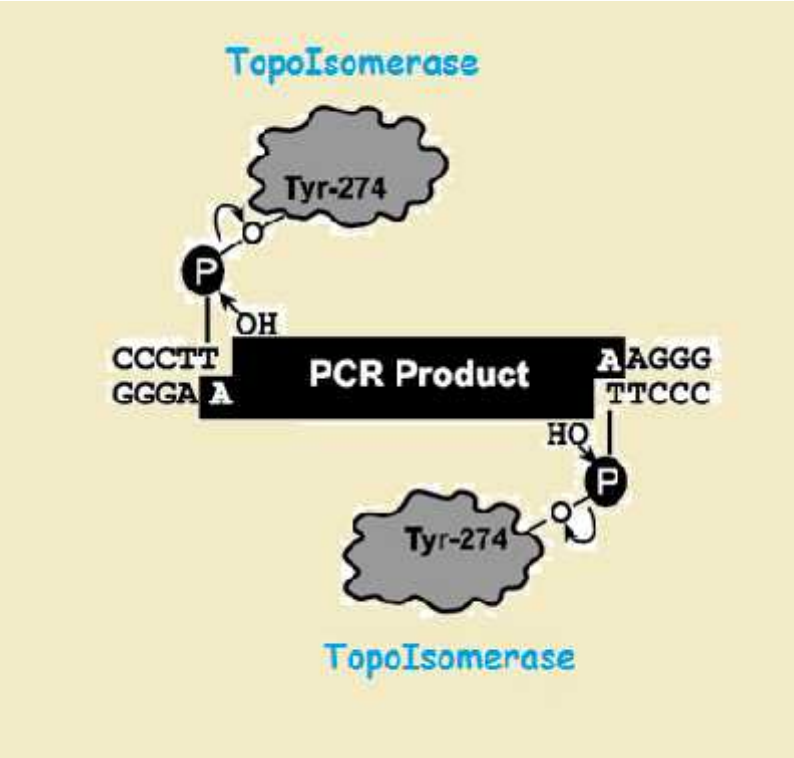


Figure 1.9 Basic mechanism of TOPO TA cloning reaction

pCR™4-TOPO® allows directly selection of recombinants by disrupting the lethal *E. coli* gene, *ccd*B. The vector contains the *ccd*B gene fused to the C-terminus of the LacZα fragment. Ligating a PCR product disrupts expression of the *lacZα-ccd*B gene fusion permitting growth of only positive recombinants upon transformation in competent cells. The following figure (**Figure 1.10**) describes the layout of the vector.

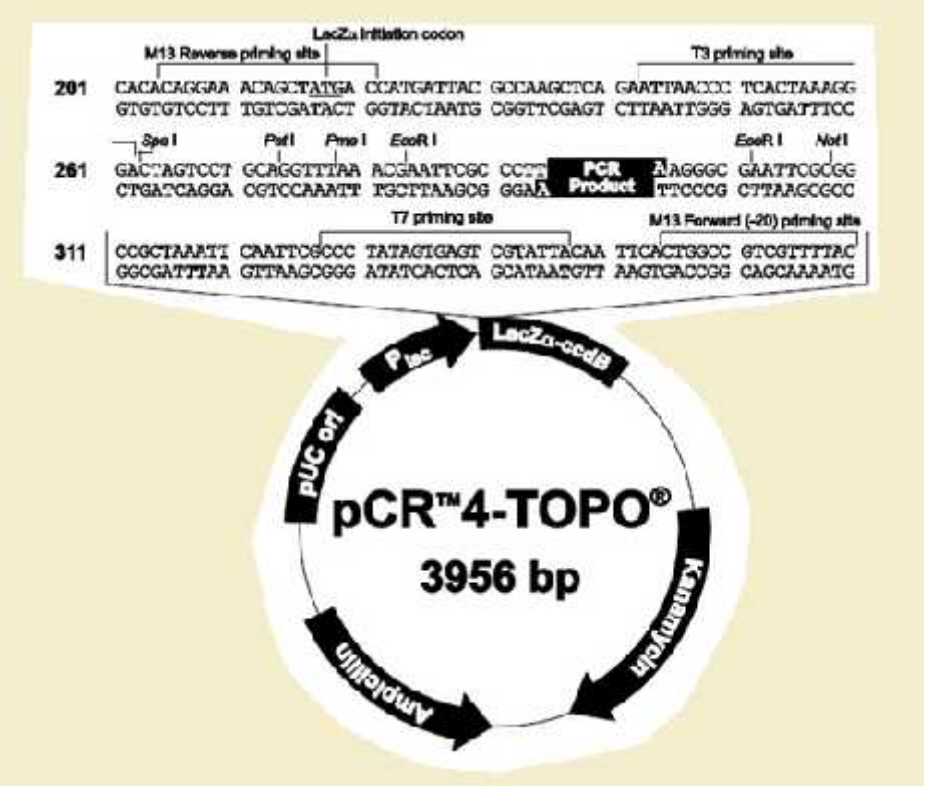


Figure 1.10 Map of pCR™4-TOPO®

1.2.4 Bioinformatics Study

1.2.4.1 Nucleotide Database

Biological database can be broadly classified into sequence and structure databases. Sequence databases are applicable to both nucleic acid sequences and protein sequences, whereas structural database is applicable to only proteins. The first database was created within a short period after insulin protein sequence was made available in 1956. GenBank. EMBL, DDBJ are for genome sequence and protein databank for protein structures. They contain information from research areas including genomics, proteomics, metabolomics, microarray gene expression, and phylogenetics (Shekhar and Ravichandran, 2007).

1.2.4.2 Sequence Alignment

Relationships between these sequences are usually discovered by aligning together and assigning this alignment a score. Popular tool for sequence alignment include

- ✓ Pair-wise alignment--BLAST
- ✓ Multiple sequence alignment—ClustalW, MUSCLE, MAFFT, and T-Coffee etc.

1.2.4.3 Construction of Phylogenetic Tree

Phylogenetic analysis has two major components

1. Phylogeny inference or "tree building" – the inference of the branching orders, and ultimately the evolutionary relationship, between "taxa" (entities such as genes, populations, species etc.)

2. Character and rate analysis for better understanding of the evolution of various traits or conditions of interest.

There are several methods available for constructing phylogenetic tree.

a. Distance-based Method- It is a method for making phylogenetic trees with DNA or protein sequences that involves calculating the percent difference between each pair of sequences and using these percent differences to construct the phylogenetic tree. Neighbour joining method is more preferable.

b. Characteristics-Based Methods- This method is said to be more powerful than distance methods because they use raw data. These methods are two types

- ✓ Parsimony searches in all possible phylogenetic trees that needs the minimum numbers of substitutions of nucleic acids or amino acids
- ✓ Maximum likelihood is the best estimates of a parameter by giving the highest probability that the observed set of measurements will be obtained.

Bootstrapping is a way of testing the reliability of a phylogenetic tree where the pseudo-replicates datasets are generated by randomly sampling the original character matrix to create new matrices of the same size as the original.

1.2.4.4 Types of Sequence Alignment

In bioinformatics, a sequence alignment is a way of arranging the sequence of DNA, RNA, or Protein to identify regions of similarity that may be consequence of functional, structural, or evolutionary relationships between the sequences (Mount, 2004).

There are mainly two types of sequence alignment

I. Global Alignment
II. Local Alignment

Global Alignment: Closely related sequences which are of the same length are very much appropriate for global alignment. Here, the alignment is carried out from beginning till end of the sequence to find out best possible alignment.

Local Alignment: Sequences which have suspected to have similarity or even dissimilar sequences can be compared with local alignment method. It finds local regions with high level of similarity.

1.3 Rationale

In Bangladesh, the main impediment in production of shrimp is the outbreak of WSSV, causing 100% mortality within 3-10 days with appearing clinical sign in shrimp, results huge economic losses every year (Hossain *et al*., 2014). In Bangladesh, an outbreak of WSSV of cultured black tiger shrimp has been found in semi-intensive farms in Cox's Bazar the south-eastern part in 1994 and quickly spreaded to Khulna the south-western part of the country. Since then shrimp farming industry in the country has been seriously affected by WSSV and shrimp productions from a unit area have also been declined. Now this disease overshadows all other disease and has been progressively increasing since 2007 (DoF, 2013).

There are no therapeutic treatments currently available for WSSV and only preventive practices can be used to manage its control. Therefore, Detection of WSSV is the main mitigation measure for this emerging disease. Currently diagonostic method for detection of WSSV include clinical observations, histopathology, dot blot, in situ hybridization, and polymerase chain reaction (PCR) methods- the last of which include conventional PCR, competitive PCR, and Real-Time PCR.(Durand and Lightner, 2002). Of these

technique, only Real-Time PCR permits the simultaneous detection and quantification of viral infection.

Many groups are involved in determining the extent of WSSV prevalence in shrimp and also in other crustaceans from several countries of the world including Bangladesh. WSSV prevalence fluctuation with seasonal variation has also been investigated at different life stages of shrimp in different countries (Iqbal *et al*., 2011).

The viral load in infected shrimp tissues is known to be one of the most important factors in the progression and transmission of the disease. Because of the continuous cell cultures for shrimp, the quantification of WSSV particles has not been possible.

Currently the genetic evolution and relationships of viruses are studied by analyzing their genetic sequence data by phylogenetic methods. Phylogenetic trees are constructed and used to deduce the genetic relatedness of the viruses. Molecular analysis of VP28 gene sequences of WSSV required to identify the change in virus strain for geographical separation.

1.4 Problem Statement

WSSV load and prevalence may vary between seasons which is related to the outbreak of WSD. Atmospheric variables, for example, water temperature, salinity, pH and rainfall may severely enhanced the propagation of WSSV and outbreak of WSD (Karim *et al*., 2012). WSSV strains found in Bangladesh may also change their genetic composition through mutation. Therefore, prevalence and quantification of WSSV between seasons is required to take measures for their control. Similarly, testing sensitivity of conventional and Real-Time PCR is required to develop sensitive and quick method for the determination of virus WSSV prevalence and their quantification.

1.5 Research Gap

WSSV appears to be more serious problem in the southwest Bangladesh than southeast (Karim *et al*., 2012). The prevalence of WSSV in that region is 79% (Hossain *et al*., 2014). However, information on seasonal variation of WSSV prevalence in cultured shrimp is completely absent in this region.Hossain *et al*. (2014) suggest that Real-Time SYBR green qPCR is required for the detection and quantification of WSSV although data onWSSV load in different season is not available in Bangladesh.Sensitivity test of different PCR technique has been done to detect the lowest viral load (Sanchez-Paz and Mendoza-Cano, 2013). Reports on sensitivity of PCR technique is also not found in Bangladesh.The diversity and geographic distribution of WSSV genetic variants increases the possibility of diagnostic failures, which may lead to dispersal of the virus (Shekhar*et al*., 2007).Therefore, sequencingBangladeshi strain is required to find out whether any genetic variation through mutation is present.

1.6 Objectives

The overall objective of this study is to improve our understanding on the seasonal occurrence, prevalence of WSSV in Satkhira district and their genomic sequencing. The specific objectives are:

1. Estimation of seasonal prevalence by Conventional PCR (targeting VP28 gene);
2. Detection and quantification of seasonal viral load of WSSV by SYBR Green based qPCR;
3. Compare the sensitivity of conventional PCR and Real-Time PCR; and
4. Molecular analysis of VP28 gene sequence of WSSV and construction of phylogenetic tree.

Materials and Methods

Chapter 2

2.1 Experimental Organism

2.1.1 Penaeid Shrimp Taxonomy and Morphology

Penaeid shrimp (*Penaeus monodon*) belong to the largest phylum in the Animal Kingdom, the Arthropoda, which is characterized by the presence of jointed appendages and an exoskeleton or cuticle that is periodically molted. The subphylum Crustacea contains about 42,000 species belonging to 10 classes. The class Malacostraca contains about three-fourths of the known species and includes crabs, crayfish, lobsters, and shrimps (Bailey-Brock and Moss, 1992).As in all Malacostraca, the body of penaeid shrimp is composed of 19 segments. Five make up the head, 8 are located in the thorax and 6 in the abdomen. The head and thorax are fused into the cephalothorax. The exoskeleton of the cephalothorax (carapace) covers the gills with a protective gill chamber and forms a dorsal keel-shaped rostrum between the eyes. The final segment is the tail fan, composed of 2 pairs of uropod and the telson.

2.1.2 WSSV morphology

WSSV is a Bacilliform non-occluded enveloped virus (Vlak *et al*., 2005). The virion consists of a rod-shaped nucleocapsid layer surrounded by a loose fitting trilaminar envelope, which consists mainly of the WSSV encoded proteins VP28 and VP19 (van Hulten *et al*., 2000). The complete WSSV genome has a circular, super coiled, and double stranded (ds) DNA, of about 300 kbp. It is one of the largest sequenced animal viral genomes available (van Hulten *et al*., 2001; Escobedo-Bonilla*et al.,* 2008)of which DNA molecule contains 59 % of AT content, that is homogeneously distributed (Tan and Shi, 2008) .

2.2 Seasons of Bangladesh

From the climatic point of view, three distinct seasons can be recognized in Bangladesh - the cool dry season from November through February, the pre-monsoon hot season from March through May, and the rainy monsoon season which lasts from June through October. The month of March may also be considered as the spring season, and the period from mid-October through mid-November may be called the autumn season (Islam *et al*., 2014)

For this study the season is divided into immediately before monsoon (mid-May to mid-June), monsoon (mid-June to mid-October) and immediately after monsoon (mid-October to mid- November).

2.3 Work Flow

The work flow for the study is given in **Figure 2.1**

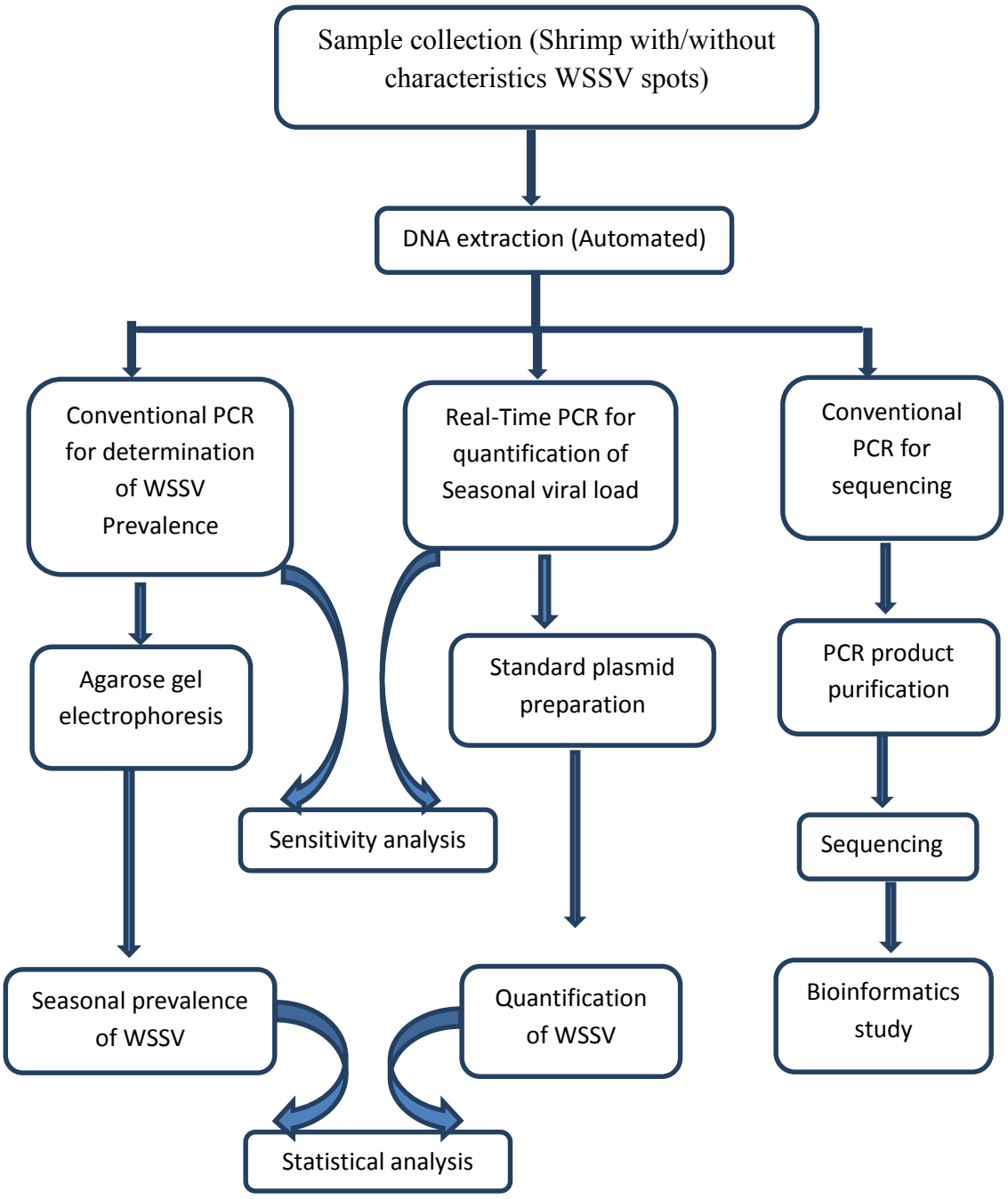


Figure 2.1 Overall work flow for the current study

All media, chemicals, reagents, and apparatus used in this work are described in appendix 5, 6 and 7 respectively.

2.4 Collection of Sample

2.4.1 Sample collection sites

Satkhira is the southernmost coastal area of Bangladesh**(Figure 2.2)**. This district covers approximately 32% area (nearly 66800 hectors out of 211000 hectors) of shrimp farms and produce about 34% (23400 metric ton out of 69000) of total shrimp production (FSYB, 2012-2013). Theprevalence of WSSV is higher (79%)than other coastal region of Bangladesh (Hossain *et al*., 2014). Therefore a study is required to determine the seasonal prevalence and viral load in this region.

Fourty eight (48) dead shrimp sample**(Figure 2.3)**, with/without characteristics spots, were collected from 4 Upazilla (Satkhira sadar, Tala, Debhata, Assasuni) of Satkhira district. Most of the shrimps displayed physical (surfacing of shrimp, erratic movement and feeding abnormalities) and phenotypic signs (White spot) of WSD.


Coastal district
Sampling area
Bay of Bengal
Bay of Bengal
Upazilla of Satkhira District
Kalaroa
Satkhira Sadar
Tala
Debhata
Assasuni
Kaliganj
Shyamnagar
Upazilla boundary
District/Country boundry

Figure 2.2 Upazilla map of Satkhira. Red mark showing the sampling sites(Inset Bangladesh)

2.4.2 History of Samples and Transportation

The specimens were collected in different season (Immediately before monsoon, Monsoon and Post-monsoon) from the cultured area. The case of WSSV was primarily diagnosed by visual appearance (white spot in the carapace). The collected samples were immediately (within 24 hours) transported from the collection site to the Laboratoryfor further analysis by maintaining cold chain (below 4ºC) effectively. In the laboratory the samples were stored at -20 ºC until tested.

Figure 2.3 Sample collected from Satkhira Sadar sampling site

2.5 DNA Extraction

2.5.1 Automated DNA Extraction

In the automated DNA extraction method 50 mg of tissue sample (below cephalothorax) was loaded into well#1 of the Maxwell® 16 SEV DNA Cartridge. Plunger was set at well#7 of the Maxwell® 16 SEV DNA Cartridge. The Maxwell® 16 DNA Cartridge contains an array of chamber facilities for DNA extraction (**Figure 2.4**). About 300 µl of elution buffer was loaded in the elution tube. After that The Maxwell® 16 SEV DNA

Cartridge and elution tube were placed in the specified chamber of the instrument. DNA extraction was carried out for 46 minutes.

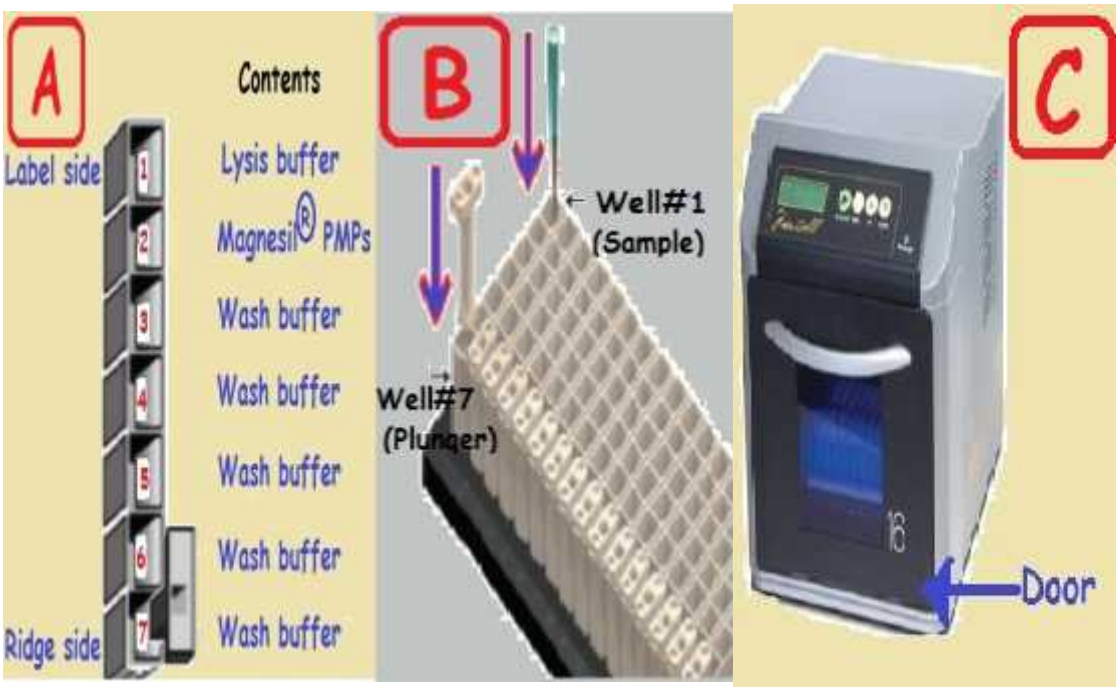


Figure 2.4 Maxwell Automated DNA extraction system. A) Maxwell ^{R} Cartidge layout B) Maxwell ^{R} Cartidge Preparation C) Maxwell 16 Extraction System

2.5.2 Measurement of the Concentration of Extracted DNA

The concentration and purity of the extracted DNA were measured by NanoDrop^{TM} 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The product was measured as ng/µl. The reading of the ratio was between at 260 nm and 280 nm (OD 260 / OD 280). This OD ratio provides an estimate the purity of nucleic acid (DNA) which is a value of 1.8.

2.6 Conventional PCR Amplification

2.6.1 Screening of WSSV

The extracted DNA was directly amplified (targeting VP28) by adding GoTaq® Hot Start Colorless Master Mix (Promega, USA). Composition of the GoTaq® Hot Start Colorless Master Mix is given in **Table 2.1.** Initial screening was done with VP28F and VP28R primers **(Table 2.2)**.

Table 2.1 Composition of GoTaq® Hot Start Colorless Master Mix

GoTaq® Hot Start Colorless Master Mix (2×)
GoTaq® Hot Start Polymerase
dNTPs (400µM each)
2× Colorless GoTaq® Reaction Buffer (pH 8.5)
$MgCl_2$(4 mM)

Table 2.2 Primer sequences used for conventional PCR amplification

Primer	Sequence (5´-3´)	References
VP28F	5´-GCGCGCGGATCCAATCATGGATCTTTCTTTCAC-3´	Namita *et al.*, 2006
VP28R	5´-GCGCGCGAATTCTTACTCGGTCTCAGTGCC- 3´	

PCR mix was prepared with the addition of 2×GoTaq® Hot Start Colorless Master Mix, 400nM of each of the primers and nuclease free water. Although GoTaq® Hot Start Polymerase component of the master mix was bound to a proprietary antibody that blocks polymerase activity at temperature below 70^{o}C, PCR mix was prepared maintaining ice-cold condition. After mixing the mixture by vortex, short centrifugation was done. After that, the reaction mix was dispensed into sterile, thin walled PCR tubes. Both positive and negative control reactions were performed to authenticate the PCR**(Table 2.3)**.

Table 2.3 PCR preparation for conventional PCR

PCR Mixture Components	Positive Control (50µl reaction)	Negative Control (50 µl reaction)	Experimental Reaction (50 µl reaction)
Nuclease Free Water	Up to 50 µl	Up to 50 µl	Up to 50 µl
GoTaq® Hot Start Colorless Master Mix (2×)	25	25	25
Upstream Primer (10 µM)	2 µl (400nM)	2 µl (400nM)	2 µl (400nM)
Downstream Primer (10 µM)	2 µl (400nM)	2 µl (400nM)	2 µl (400nM)
Template	Variable (< 500 ng)	No Template	Variable (< 500 ng)

All the PCR tubes containing the appropriate mixtures were heated at 95 ºC for 4 minutes in the thermal cycler (Applied Biosystem, USA) to ensure denaturation of all DNA templates. Thirty (30) cycles of these segments were repeated with a final extension of 5 minutes at 72ºC. After this, PCR tubes were stored at -20 ºC until further analysis. The cycling profile for each primer: target combination was optimized accordingly **(Table 2.4)**

Steps	Temperature and Time Profile

Table 2.4 PCR profile for conventional PCR

Denaturation	95 ºC for 60 seconds
Annealing	55 ºC for 30 seconds
Extension	72 ºC for 45 seconds

2.6.2 Agarose Gel Electrophoresis

The amplified products were run on 1 % agarose gel with a 1kb-DNA ladder (Promega, USA) for visualization of the amplified products. Agarose (Agarose LE, Analytical grade, Promega, Spain) was measured at an amount of 0.60 g and mixed with 60 ml 1x TAE buffer **(Appendix 6)** to prepare 1% agarose gel (Appendix 6). The mixture was heated in microwave (details) for ~5 min on medium until the agarose melted. Then the boiled mixture was allowed to cool to about 45°C before 3µl Ethidium bromide (stock 10 mg/ml) was added. The gel was poured onto gel casing and well former (comb) was inserted. The casing was then allowed to set on a flat surface for about 15 min. 1x TAE Buffer was then poured into the electrophoresis tank and comb was removed from gel. Samples were mixed with loading dye on parafilm (1µl loading buffer and 5µl PCR product). Molecular weight marker was prepared by mixing 6µl molecular weight marker and 1µl loading buffer (Appendix 6). Samples were loaded into the wells formed in the gel using sterile tips. Electrophoresis was set at 100 volts for 35 min. The gel was viewed on AlphaImager HP Gel-documentation system (Cell Bioscience, USA).

2.6.3 DNA Quality Test

For testing the extracted DNA was run in 2 % agarose gel with a 1kb-DNA ladder (Promega, USA). Details in **section 2.6.2**.

2.7 PCR Purification

2.7.1 Purification of Amplicon

After Agarose gel electrophoresis the PCR positive sample was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA; Appendix 6). Centrifugation based methodology was followed. The Wizard® SV Gel and PCR Clean-Up System are based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts (guanidine isothiocyanate). After amplification, an aliquot of the PCR was added to the guanidine isothiocyanate containing Membrane Binding Solution (MBS) and directly purified. An equal volume of MBS was added to PCR amplification. The mixture was transferred to the mini column pre-set with a collection tube (SV mini column assembly). After short (2 minutes) incubation at room temperature, SV mini column was centrifuged at 16,000×g (14,000rpm) for 1 minute. After discarding the flow-through, SV mini column was subjected to wash for two times with Membrane Wash Solution (Supplied in the kit, ethanol added). After washing the SV mini column, DNA was eluted in Nuclease Free Water (Supplied in the kit). The purified plasmid was stored at -20^{o}C until further processing.

2.7.2 Measurement of the Concentration of the Amplicon

The amount of PCR product was measured according to the protocol described in chapter **2.5.2**

2.8 Preparation of Recombinant Plasmid DNA Based Standard for Real-Time PCR

The recombinant plasmid was constructed using the insert VP28 primer amplified 643 bp PCR product with a linearized plasmid vector, pCR™4-TOPO® supplied in the kit (TOPO® TA Cloning® Kit, Invitrogen, USA) in a ligase independent manner**(Figure 2.5)**. Competent cell was not supplied in the kit, so preparation of competent cell was included in the cloning procedure.

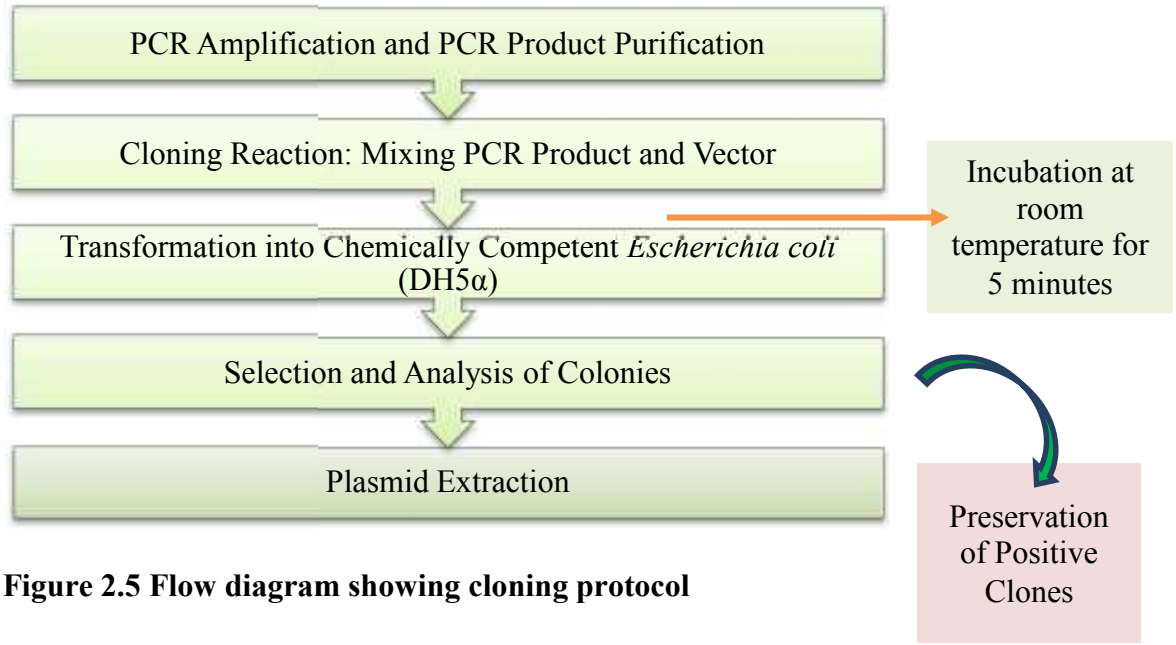


Figure 2.5 Flow diagram showing cloning protocol

2.8.1 Competent Cell Preparation

Competent cell was prepared by chemical method using *Escherichia coli* DH5α. Frozen glycerol stock of bacterial cells (DH5α) was streaked on the nutrient agar **(Appendix 5)** followed by overnight incubation at 37ºC. Antibiogram of the organism was done against Ampicillin and Kanamycin because selection marker of the vector was resistance to both of the antibiotics. After that an isolated colony was inoculated on 5 ml Luria-Bertani **(Appendix 5)** broth and incubation at 37ºC in a shaker incubator (130 rpm) until saturation. Then 100 µl of the saturated culture was transferred to another fresh 10 ml LB broth and incubated at shaker incubator (37ºC, 130 rpm) until OD reached 0.35-0.40. After that culture was split into four 1.5 ml microcentrifuge tubes and chilled on ice for 30 minutes. The cells were harvested by centrifugation (3000 × g, 4-5 min, 4ºC). After centrifugation, the supernatant was discarded followed by ice-cold 0.1 M $MgCl_2$ wash (0.4 volume of 0.1 M $MgCl_2$). After wash, cells were harvested by centrifugation (3000×g, 4-5 minutes, 4º C). Discarding the supernatant, the pellet was dissolved in 0.8 volume of ice-cold 0.1 M $CaCl_2$ followed by 30 minutes incubation on ice. Again cells were harvested by centrifugation (2000 × g, 4 min., 4ºC) and finally after discarding the supernatant, the pellet was dissolved in 0.1 M ice-cold $CaCl_2$. The preparation was kept on ice overnight because the cells are best competent in the day after preparation.

Usually competent cells were prepared before every cloning reaction, so there was no need of long term storage.

2.8.2 PCR for Cloning

Genomic DNA extracted from shrimp tissue was used as template. PCR reaction mixture was prepared with the combination of 2× GoTaq® Hot Start Colorless Master Mix (**Table 2.1**), 400nM of forward and reverse primer and nuclease free water. The PCR reaction was prepared following the protocol described in **Table 2.3**. PCR reaction mixture was mixed with template DNA and heated at 95ºC for 4 minutes followed by 95º C for 60 seconds, 55ºC for 30 seconds and 72ºC for 45 seconds. Final Extension was set at 72ºC for 5 minutes. Amplified PCR products were resolved according to the protocol described in **section 2.6.2**. Gel purification of the desired amplicon (643 bp) was done according to the procedure in section **2.7.1**

2.8.3 Cloning Reaction

Gel purified PCR product was subjected to TA cloning reaction. Vector only control cloning reaction was performed in parallel. The cloning reaction can be deciphered as follows:

i. The cloning reaction was set up (as per **Table 2.5**).
ii. The reactants were mixed gently.
iii. The reactants were incubated at room temperature for at least 5 minutes.
iv. Then the reaction was kept in ice until transformation was carried out.

For insert >1kb in length, incubation at room temperature was done for more than 5 minutes

Table 2.5 Set up of the cloning reaction

Reagent	Vector Only	Vector + Insert
PCR product	-	4 µl
Water	4 µl	-

Salt solution (1.2M NaCland 0.06M $MgCl_2$)	1 µl	1 µl
pCR™ 4-TOPO®(Vector)	1 µl	1 µl

2.8.4 Chemical Transformation

Transformation of the chemically competent *Escherichia coli* DH5α was done with recombinant plasmid constructed by cloning reaction. About 3µl of the TOPO TA cloning reaction was added into a vial containing cells of chemically competent *E. coli* (50 µl) and mixed gently. The mixture was incubated on ice for 5-30 minutes. After that, cells were subjected to heat shock at 42ºC for 30 seconds without shaking followed by immediate transfer on ice for another 2 minutes. About 450µl of the pre-warmed S.O.C. medium **(Appendix 5)** was added into the microcentrifuge tube containing competent cell after heat shock. The microcentrifuge tubes were capped tightly and incubated at 37ºC shaker incubator (horizontal shaking, 200 rpm) for 1 hour. After that, brief centrifugation was done to collect the cells at the bottom of the micro centrifuge tubes. About 10–100µl of transformed cells were spread on a pre-warmed selective plate (Kanamycin, 50µg/ml). For plating small volume (< 20 µl) of transformed cells fresh pre-warmed S.O.C. medium was used as diluent to facilitate spreading. Plates were incubated at 37ºC in the incubator for subsequent screening.

2.8.5 Screening of Transformants

The day after transformation (usually 18 hours after plating transformation reaction on LB agar containing kanamycin) the colonies that appeared on the plates were analyzed for transformants. Initially patch of all positive clones were done on another fresh media (LB agar containing kanamycin; **Appendix 5**). Plates containing patches was incubated at 37ºC overnight. Initial screening for transformants was done by means of colony PCR. About 10 µl of PCR reaction mixtures were prepared by addition of 2×GoTaq® Hot

Start Colorless Master Mix, 400nM of each of the primers (**Table 2.6**) and Nuclease free water. A single colony was re-suspended in the dispensed PCR reaction mixture containing tubes. All the PCR tubes containing the appropriate mixtures were heated at 94ºC for 10 minutes in the thermal cycler (Applied Biosystems, USA) to lyse the cells and denaturation of plasmid DNA templates. After that 35 cycles at 94ºC for 1 minute, 48ºC for 1 minutes and 72ºC for 1 minutes 30 seconds was done followed by final extension at 72ºC for 7 minutes. Amplified PCR products were visualized according to protocol described in **section2.6.2.** PCR positive clones were inoculated in LB broth containing 100µg/ml Kanamycin (Appendix I) and incubated at 37ºC overnight (130 rpm).

Table 2.6 Primers used for screening transformants

Primer	Sequence (5´-3´)	Annealing (ºC)	Product (bp)
T3	ATTAACCCTCACTAAAGGGA	48	643 + insert
T7	TAATACGACTCACTATAGGG		

2.8.6 Plasmid Extraction

Plasmid DNA was extracted from the saturated overnight culture using PureYield™ Plasmid Miniprep System (Promega, USA; Appendix 6). Centrifugation based methodology was followed when purification of the plasmid DNA was conducted. About 1ml bacterial culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at maximum speed (16000 x g) for 30 seconds. The supernatant was discarded. The same step was repeated 2 times. The pellet was suspended in 600µl nuclease free water. About 100µl of the cell lysis buffer was added into the tube and mixed by inverting 6 times which turned the color of the mixture blue. After that, 350µl cold neutralization buffer (4-8ºC) was added into the tube and mixed by inverting until color of the mixture turned yellow (complete neutralization). Then centrifugation at maximum speed (16000xg) in a microcentrifuge was done for 3 minutes followed by transfer of the supernatant into Pure

Yield™ Mini column assembled with collection tube without disturbing the cell debris pellet. Centrifugation at maximum speed for 15 seconds was done and flow-through was discarded. About 200µl of Endo toxin Removal Wash was added into the mini column and centrifugation at maximum speed for 15 seconds was done. After that 400µl of the Column Wash Solution was added to the mini column and centrifugation at maximum speed in a micro centrifuge for 30 seconds was done. Mini column was transferred into 1.5 ml clean microcentrifuge tube and 30µl of nuclease-free water was added directly to the mini column matrix followed by incubation at room temperature for 1 minute. Centrifugation at maximum speed for 15 seconds was done to elute plasmid DNA and was stored at -20ºC until further processing.

2.8.7 Preservation of Positive Clones

Original clones were preserved for long term use. About 0.85 ml of saturated culture of positive clones in LB broth containing 100µg/ml Kanamycin was mixed with 0.15ml sterile molecular biology grade glycerol (Promega, USA) and transferred into Cryovial. Vials were stored at -80º C.

2.8.8 PCR for Conformation of Recombinant Plasmid

Conventional PCR using VP28 primer was carried out to reconfirm that the extracted plasmid is the recombinant plasmid which was transformed into *Escherichia coli* DH5α according to the protocol described in **section 2.6.1.**

2.8.9 Separation of Plasmid DNA by Agarose Gel Electrophoresis

Extracted plasmid after being confirmed that this is the recombinant one was separated by gel electrophoresis in 2% low melting agarose (L.M.T) slab gel in a Tris-acetate EDTA (TAE) buffer at room temperature using 70 volt (50 mA) for 1 hour. Low melting agarose was prepared by adding 1.2g analytical grade low melting agarose in 60ml TAE buffer and heating upto boiling. The gel was poured in the gel casing and kept for solidification. After 30 minutes the gel was placed in a refrigerator at 4°C for 30 minutes. About 30µl of plasmid DNA was mixed with 5µl of loading buffer (Appendix-6) and was loaded into the individual well of the gel. The gel was stained with Ethidium bromide for 30 min at room temperature. DNA bands were visualized and photograph was taken using Gel Doc (AlphaImager, USA). The molecular weight of the unknown

plasmid DNA was determined on the basis of its mobility through agarose gel and was compared with the mobility of the known molecular weight plasmids of *Escherichia coli* V_{517}.

2.8.10 Gel Purification of Recombinant Plasmid

After electrophoresis in low melting agarose gel (UltraPure™L.M.P. Agarose, Spain), the gel was visualized in UV and gel containing recombinant plasmid of approximately 4599 bp was cut and taken into an eppendorrf tube and weighed to measure. The gel was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The Wizard® SV Gel and PCR Clean-Up System are based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts (guanidine iso-thiocyanate). About 10µl of Membrane Binding Solution per 10mg of gel slice was added and incubated at 50–65°C until the gel slice is completely dissolved. After the gel is melted the mixture was transferred to the mini column pre-set with a collection tube (SV mini column assembly). After short (2 minutes) incubation at room temperature, SV mini column was centrifuged at 16,000×g (14,000rpm) for 1 minute. After discarding the flow-through, SV mini column was subjected to wash for two times with Membrane Wash Solution (Supplied in the kit, ethanol added). After washing the SV mini column, DNA was eluted in Nuclease Free Water (Supplied in the kit). The purified plasmid was stored at -20°C until further processing.

2.8.11 Further Confirmation of Gel Purified plasmid

Further confirmation of gel purified plasmid was performed by setting a conventional PCR with VP28F and VP28R primer and reaction condition was 95ºC for 4 minutes (initial denaturation) followed by 95ºC for 60 seconds (cycle denaturation), 55ºC for 30 seconds (annealing) and 72ºC for 45 seconds (extension). Final Extension was set at 72ºC for 5 minutes.

2.9 Real-Time PCR Assay

2.9.1 Recombinant Plasmid DNA Based Standard Curve

Recombinant plasmid was prepared and extracted which is described in **section 2.8** and the concentration of recombinant plasmid was measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). PCR product was measured as ng/µl.

2.9.1.1 Determination of mass of recombinant plasmid DNA

Mass of the recombinant plasmid DNA can be calculated by using the formula,

$m = [n]\ [(1/6.023\times10^{23})]\ [660]$ g

Here,

n = Size of recombinant plasmid DNA (bp)

m= mass of plasmid DNA

Avogadro's number= 6.023×10^{23} molecule/mole

Average molecular weight of double stranded DNA is 660 g/mole.

The simplified expression is, $m = [n]\ [1.096\times10^{-21}]$ g

In this experiment, Size of the vector was 3956 bp and size of the insert was 643 bp.

So, size of the total recombinant plasmid DNA was 4599 bp

So, the mass of single recombinant plasmid is, **$m= 4599 \times [1.096\times10^{-21}]$ g**

$= 5.040504 \times 10^{-18}$ g

$= 5.040504 \times 10^{-9}$ ng ($1\ ng = 10^{-9}$ g)

2.9.1.2 Preparation of Dilution Series

The VP28 gene is a target that exists as a single copy gene per plasmid vector. Therefore, 5.040504×10^{-9} ngof recombinant plasmid DNA contains one copy of the VP28 gene. Therefore, copy number of interest × mass of recombinant plasmid DNA = mass of recombinant plasmid DNA needed. The mass of recombinant plasmid DNA needed was divided by the volume to be pipetted into each reaction to get the final concentration. Calculations about preparation plasmid DNA dilution is shown in **Table 2.7**

Table 2.7 Dilutions series of recombinant plasmid DNA (2.5 µl of template DNA per PCR reaction)

Copy number of VP28 gene	× mass of recombinant plasmid DNA (ng)	Mass of recombinant plasmid DNA needed (ng)	Amount of DNA to be pipetted	Final concentration (ng/µl)
10^9	5.040504×10^{-9}	5.040504	2.5 µl	2.0162016
10^8	5.040504×10^{-9}	0.5040504	2.5 µl	0.20162016
10^7	5.040504×10^{-9}	0.05040504	2.5 µl	0.020162016
10^6	5.040504×10^{-9}	0.005040504	2.5 µl	0.0020162016
10^5	5.040504×10^{-9}	0.0005040504	2.5 µl	0.00020162016
10^4	5.040504×10^{-9}	0.00005040504	2.5 µl	0.000020162016
10^3	5.040504×10^{-9}	0.000005040504	2.5 µl	0.0000020162016
10^2	5.040504×10^{-9}	0.0000005040504	2.5 µl	0.00000020162016
10^1	5.040504×10^{-9}	0.00000005040504	2.5 µl	0.000000020162016

2.9.1.3 Making Serial Dilution

A serial dilution of the plasmid DNA was prepared using the following formula:

$C_1V_1 = C_2V_2$

For example, each dilution prepared has a final volume of 10 µl (V_2)

Dilution no.1

Final DNA concentration, C_2 = 2.0162016 ng/µl

Initial DNA concentration, C_1 = 11.8 ng/µl

Volume to be taken from stock, V_1 = [10 × 2.0162016]/ 11.8= 1.7 µl

Nuclease free water to be added to achieve Dilution 1= (10-1.7) or 8.3 µl.

Dilution 2 to Dilution 7 was prepared like the protocol described in case of Dilution1 and **Figure 2.6** is shows preparation of dilution for plasmid DNA based standard and **Table 2.8** is showing dilution series of plasmid DNA.

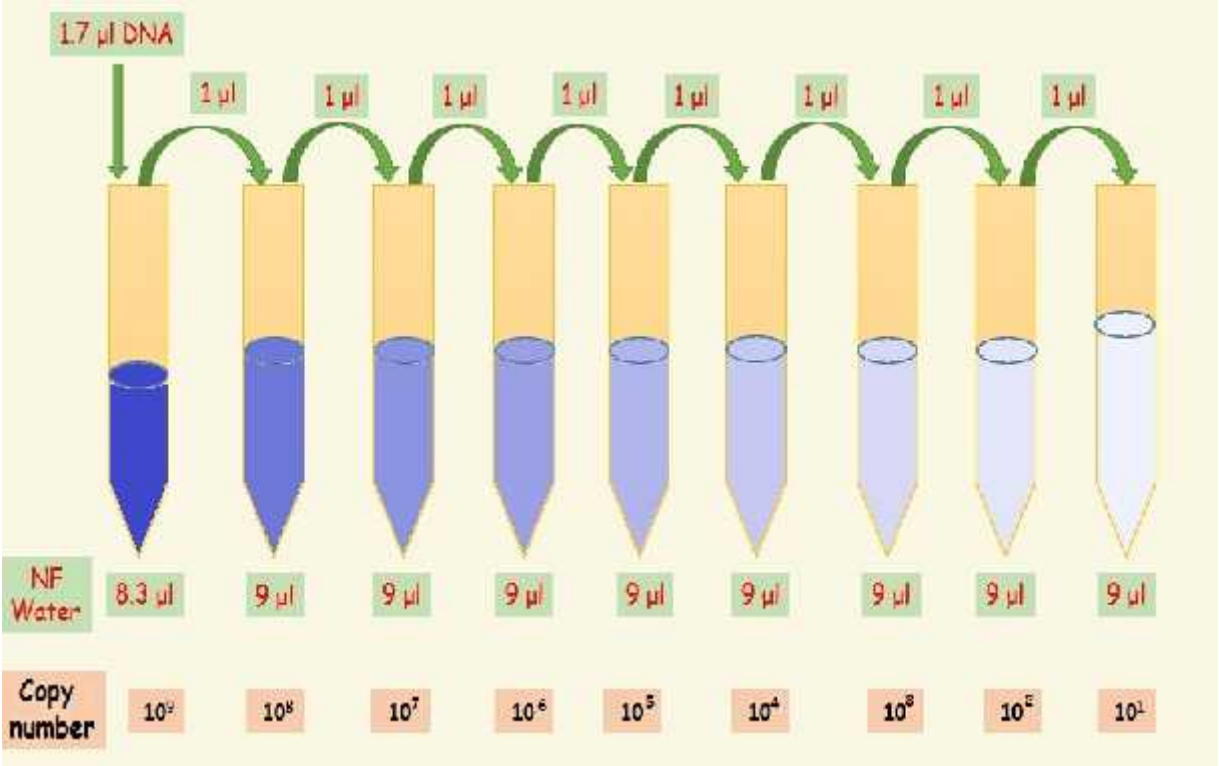


Figure 2.6 Dilution of plasmid for standard curve preparation

Table 2.8 Final concept of dilutions

Dilution	Source of DNA for Dilution	Initial conc. (ng/µl)	Volume of Plasmid DNA (µl)	Volume of diluent (µl)	Final conc. (ng/µl)	Final Volume (µl)	Resulting copy of VP28 gene/ 2.5µl
1	Stock	11.8	1.7	8.3	2.0162016	10	10^9
2	Dilution 1	2.0162016	1.0	9.0	0.20162016	10	10^8
3	Dilution 2	0.20162016	1.0	9.0	0.020162016	10	10^7
4	Dilution 3	0.020162016	1.0	9.0	0.0020162016	10	10^6
5	Dilution 4	0.0020162016	1.0	9.0	0.00020162016	10	10^5
6	Dilution 5	0.00020162016	1.0	9.0	0.000020162016	10	10^4
7	Dilution 6	0.000020162016	1.0	9.0	0.000002016 2016	10	10^3
8	Dilution 7	0.000002016 2016	1.0	9.0	0.000000201 62016	10	10^2

2.9.2 Quantitative Real-Time PCR Assay

2.9.2.1 Preparation of Reaction Mixture

For preparation of standard curve, calculations were performed according to the previous **section 2.9.1.1** and standard dilutions were prepared using the plasmid DNA extracted by the method described in **section 2.8**. After standard dilution preparation, reaction mix was prepared. During reaction mixture preparation SYBR green master mix (SYBR Green, Applied Biosystems, Korea) (2.0×) was used whose final concentration was found to be 1.0×. Forward and reverse primer(previously designed in our lab) had a stock concentration of 10 µM and working concentration of 100nM**(Table 2.9)**.

Table 2.9 Primer pair used for Real-time quantification

Primer	Sequence (5′-3′)
WSSV-q28F	5′-TGTGACCAAGACCATCGAAA-3′
WSSV-q28R	5′-CTTGATTTTGCCCAAGGTGT-3′

A reaction mix was prepared by adding all the components described in **Table 2.9** (except template DNA) for each 25µl reaction to a tube at room temperature. All solutions were gently vortexes and briefly centrifuged after thawing. The master mix was mixed thoroughly and dispensed in appropriate volumes into well of Micro Amp™ Optical 8-tube Strip, containing 22.5µl of reaction mixture without template DNA. Template DNA (10 fold dilution from the extracted DNA) was added to a volume of 2.5µl/reaction to the individual PCR tubes containing the master mix. Each strip was sealed properly with Micro Amp™ Optical 8-Cap Strip. The reactions were mixed by gentle centrifugation without creating bubbles after addition of template. Assay mix was kept protected from light, in the freezer, until use. Excessive exposure to light may affect the fluorescent dye.

Table 2.9 Reaction mix preparation

Components	Volume for single reaction
Master mix	12.5 µl
Forward primer qVP28F	0.25 µl
Reverse primer qVP28R	0.25 µl

Nuclease free water	9.5 µl
Template	2.5 µl
Total volume	25 µl

The target VP28 was defined in the software and the reporter was defined as SYBR green and there was no quencher present in the assay. The name of the sample was defined and each was assigned a position. Each reaction was present in duplicate having copy number from 10^9 to 10^2 copy DNA and two no template control was included in the assay.7500 software, ver. 2.0.6 (Applied Biosystems, Foster City, CA, USA) was used for data analysis.

2.9.2.2 Reaction Condition and Melt Curve of Real-Time PCR

Reaction condition in real time PCR assay was 50ºC for 2 minutes followed by 95ºC for 10 minutes, followed by 40 cycles at 95ºC for 15 seconds and 55ºC for 30 seconds as well as melt curve analysis.

2.9.3 Quantification of WSSV

As volume of template is limited to 10% to the reaction volume using this ABI 7500 software, in a reaction of 25 µl, 2.5 µl template DNA is added.

Viral load per g of tissue was calculated by using this formula

Viral load per 50 mg sample = viral load × (Final elution volume /Template volume

= viral load per reaction × (300µl / 2.5 µl)

= viral load per reaction × 120

Viral load per g sample = viral load per reaction × 120 × 20

= viral load per reaction ×2400

Where dilution was done the formula was changed as follows

Viral load per g sample = viral load per reaction × 2400 × Dilution factor

= viral load per reaction × 24000 (Dilution factor= 10)

In Real Time PCR assay, a duplicate or triplicate of sample and standard was performed to make the result statistically more valid and significant.

So, in case of duplicate or triplicate run of same sample the formula was expressed **viral load per g sample ± Standard deviation**

2.10 Sensitivity Analysis

The dilution series (tenfold) prepared described in **section 2.9** was used for sensitivity analysis. The conventional PCR (by using WSSV-q28 primer pair) was used to amplify VP28 gene. The PCR was performed according to the protocol described in previous section section. Where conventional PCR failed to detect WSSV in tenfold dilution, from there two fold dilution was made. The conventional PCR and the SYBR green qPCR was simultaneously performed to determine which has the highest sensitivity.

2.11 Sequencing Based Identification

2.11.1 Sequencing Reaction

For confirmation of the PCR products of VP28, cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA) according to the manufacturer's instruction. Extension product was purified followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystems®, USA). The basic sequencing protocol is illustrated below.

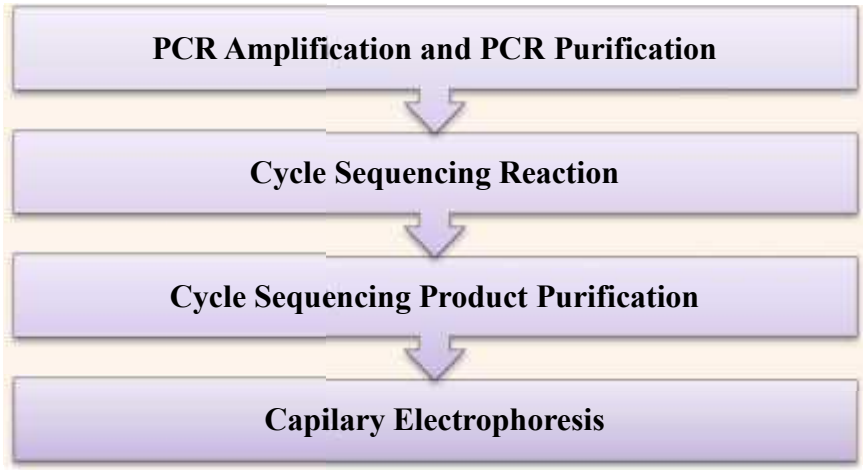


Figure 2.7 Sequencing of PCR product of VP28

2.11.2 Sequence Alignment and Identification

Partial sequences of desired genes obtained using specific forward and reverse primers were combined to full length sequences using the SeqMan Genome Assemblerand were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) by means of the basic local alignment search tool (BLAST) to identify their close phylogenetic relatives.

2.12 Phylogenetic Analysis

2.12.1 Construction of Phylogenetic tree

Phylogenetic tree of the VP28 gene sequences of the WSSV was constructed using the following servers and bioinformatics software:

A. Reference sequences were downloaded from

NCBI: http://www.ncbi.nlm.nih.gov

B. Partial sequences, obtained using forward and reverse primers, were combined to full length sequences via the SeqMan Genome Assembler and then aligned, checked and processed by using Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura *et al*., 2013), an integrated tool for conducting sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses.

C. Phylogenetic tree was constructed by MEGA 6.

Briefly, the multiple sequence alignment of the retrieved reference sequences from NCBI and sequences of VP28 gene was performed with the ClustalW program embedded in Mega 6. Aligned sequences were refined by sequence trimming and conserved region identification. Refined sequences were used for selecting best model and phylogenetic tree construction using the Neighbor-Joining Algorithm and selecting 1000 bootstrap replication. Further analysis of the genes was carried out using the Distance and Pattern analysis tools in the MEGA software.

2.12.2 Detection of Amino Acid Substitutions in VP28

The forward and reverse sequences of each isolate were assembled using SeqMan Genome Assembler (Swindell and Plasterer 1997) and the contig sequence was saved. The contig sequences were aligned with the deposited sequences at GenBank database (Benson *et al*. 2005) of NCBI using Basic Local Alignment Search Tool (BLAST) and the sequences were refined using SeqMan Genome Assembler. The nucleotide sequences were converted into corresponding amino acid sequences of all six frames using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) online tool. The amino acid sequence of right reading frame corresponding to each nucleotide sequence was selected by homology search using protein BLAST. The reference amino acid sequences were retrieved from the GenBank database and were aligned with my sequences using BioEdit Sequence Alignment Editor to find the amino acid substitutions in query sequences.

2.13 Data Analysis

Viral load data were transformed into natural log transformations. 2-way anova was applied to analyze the data between seasons and sampling sites. Data between sampling sites were compared by 1-way followed by Tukey's HSD post hoc for multiple comparisons. Data were presented as mean ± 1 SEM. Level of significance considered in this study was p at 0.05.

Results

Chapter 3

3.1 Amplification of VP28 gene with VP28F/VP28R Primer Pair

VP28F/VP28R targets portion of WSSV genome (VP28) yielded an amplicon of about 643bp. The primer pairs identified 40 positive samples of 48. The representative gel autoradiograph of the amplicon is delineated in **Figure 3.1**

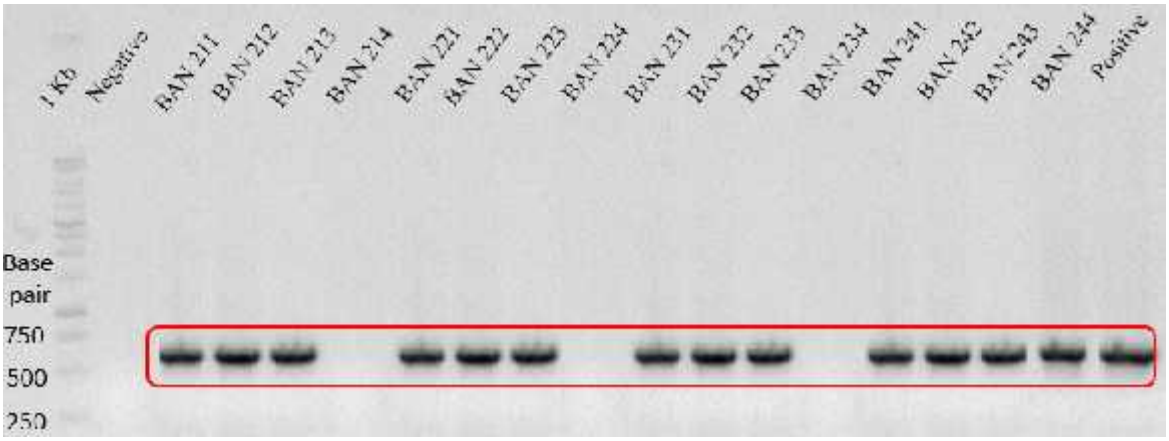


Figure 3.1 PCR amplification products of VP28. Amplicon size was 643 bp. Here 1 kb (Promega, USA) marker was used. Sample ID is given Appendix 1.

3.2 Assessment of the Quality of the DNA

The PCR negative sample and some representative PCR positive sample further analyzed to conform whether genomic DNA was present or not in that sample. The result showed that in all the sample DNA was present. A representative gel autoradioradiograph is given in **Figure 3.2.**

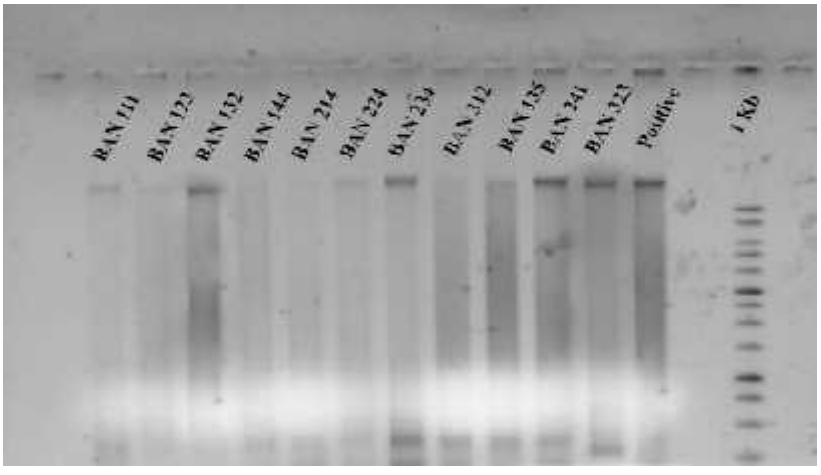


Figure 3.2 Gel picture showing the DNA quality. Here 1 Kb (Promega, USA) marker was used. Sample ID is given in Appendix 1.

3.3 Standard Curve preparation

3.3.1 Recombinant Plasmid DNA Standard

Recombinant plasmid containing 643 bp VP28 amplicon was created and further transformed in to competent cells (Figure 3.3).

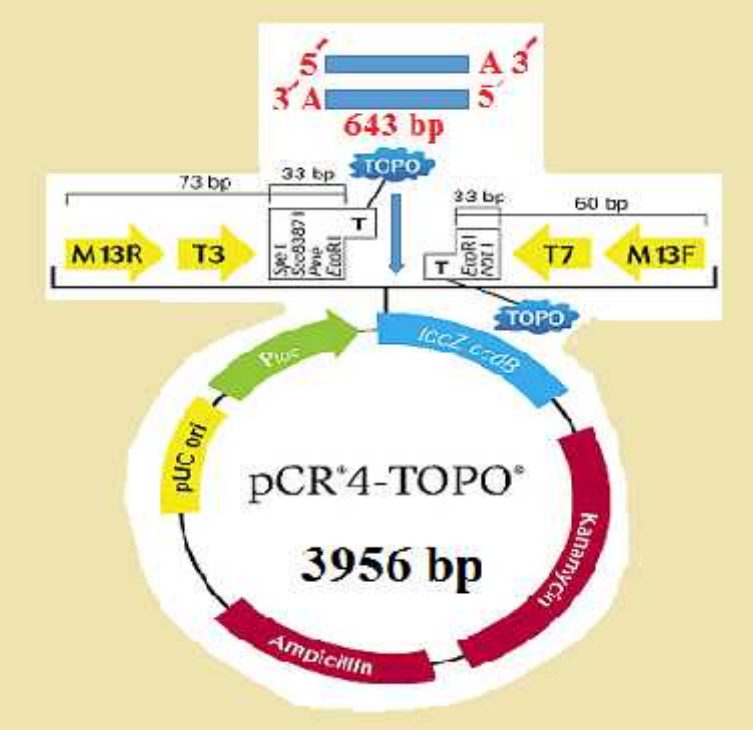


Figure 3.3 Schematic figure of 643 bpamplicon for cloning reaction and recombinant plasmid construction for transformation.

3.3.2 Screening of Transformants

Positive transformants were selected by colony PCR using T3:T7 primer pair. Amplicon size was 106 bp larger than the size of insert. Approximately 750 bpamplicon was found in the gel electrophoresis. A representative gel autoradiograph is given in **Figure 3.4**.

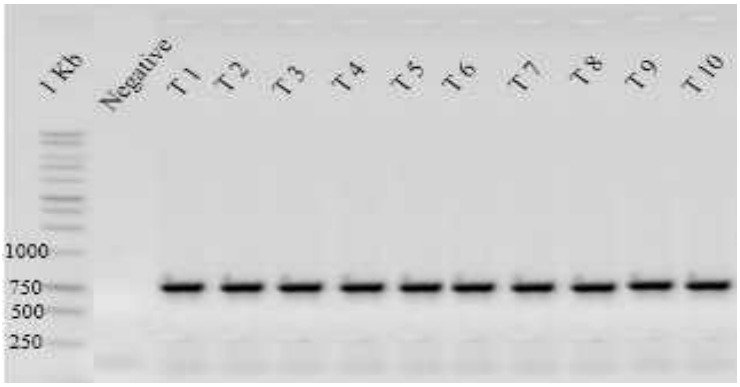


Figure 3.4 Representative gel autoradiograph showing amplicon of T3:T7 primer pair. Here transformants carrying cloned plasmid are shown. Amplicon size about bp [643+106] and marker used was 1 kb DNA ladder (Promega, USA).

3.3.3 PCR for Confirmation of Recombinant plasmid

Conventional PCR using VP28 primer reconfirmed that the extracted plasmid was the recombinant plasmid which was transformed into *Escherichia coli* DH5α. Amplicon size was 643 bp. A representative gel autoradiograph is given in **Figure 3.5.**

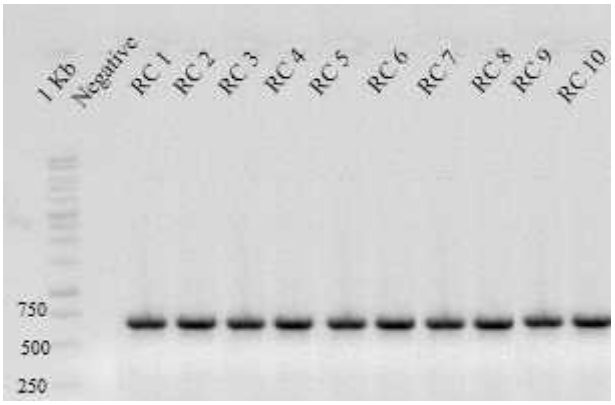


Figure 3.5 Representative gel autoradiograph showing amplicon of VP28 primer pair. Here transformants carrying recombinant cloned plasmid are shown. Amplicon size was about 643bp and 1 kbmarker used (Promega, USA).

3.3.4 Confirmation of Gel Purified Plasmid

Conventional PCR with VP28primer pair reconfirmed that gel purified plasmid was the desired one **Figure 3.6**.

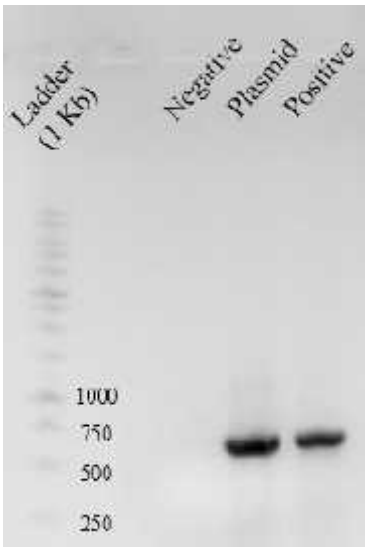


Figure 3.6 Representative gel autoradiograph showing amplicon of VP28 primer pair in gel purified plasmid. Amplicon size was about 643bp and 1 kbmarker used (Promega, USA).

3.4 Prevalence of WSSV by Qualitative Conventional PCR

Qualitative estimation of WSSV prevalence was performed with Conventional PCR between the collected cultured shrimp sample. Appendix 1 shows the percentage of WSSV prevalence in the immediate before monsoon, during monsoon and immediate after monsoon seasons from Satkhirasadar, Tala, Debhata, Assasuni of Satkhira district.

3.4.1 Seasonal Prevalence

The cumulative WSSV prevalence was the lowest (75%) recorded during immediate before monsoon season among all four locations of Satkhira, whereas the highest WSSV prevalence of 93.75% was found during the immediate after monsoon season. Notably, during the monsoon season, a moderate WSSV prevalence (81.25%) was found **(Figure 3.7)**

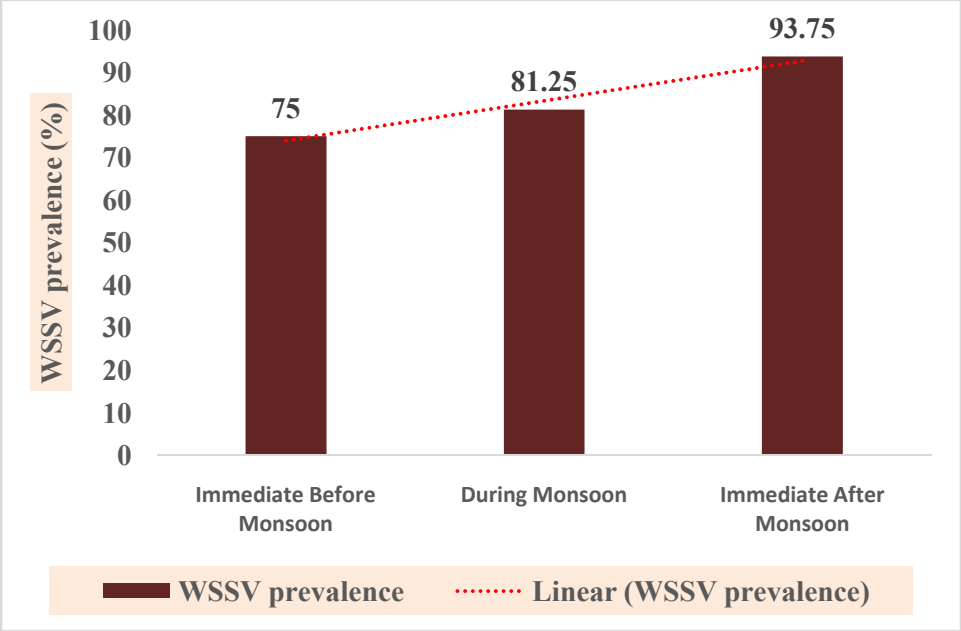


Figure 3.7 Cumulative distribution of WSSV prevalence during different seasons of Satkhira district

3.4.2 Spatial prevalence

These results showed that, starting from the northernmost location of this study, Satkhirasadar to the southernmost location, Assasuni, WSSV prevalence increased. At Satkhirasadar, WSSV prevalence was as low as 75%, which increase at Tala and Debhata (83%), whereas the highest (91%) prevalence was found at Assasuni**(Figure 3.8)**.

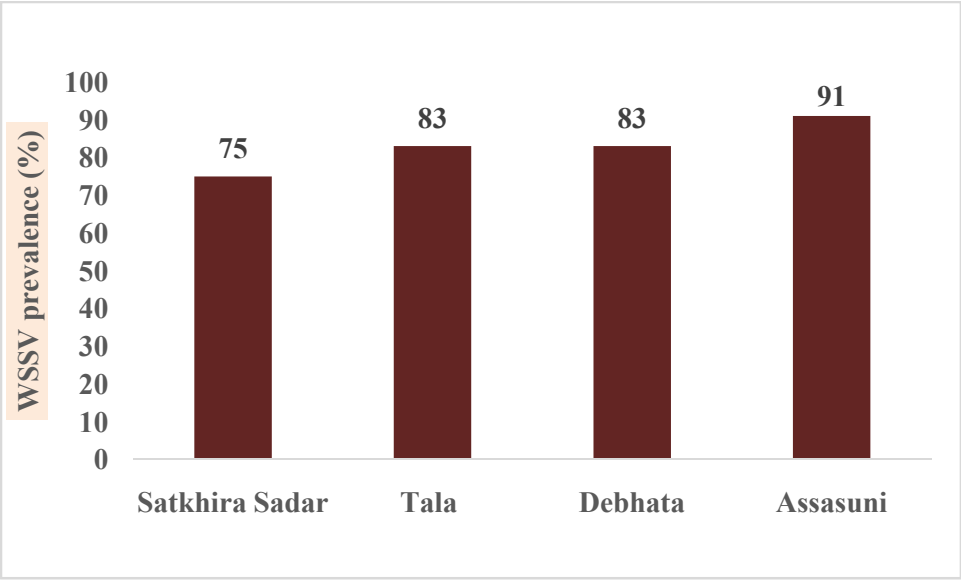


Figure 3.8 Cumulative WSSV prevalence in four location across the seasons

3.5 Viral copy number of WSSV with Quantitative Real-Time PCR

3.5.1 Real-Time PCR Assay

Gel purified plasmid was serially diluted to make a dilution series from 10^9 to 10^2. The lower detection limit was 100 copies having a C_T mean value of 38.824 and standard deviation was also calculated by the software 7500 software, ver. 2.0.6 (Applied Biosystems, Foster City, CA, USA) and found to be 0.124. The quantity of lowest amount of DNA which can be measured by the real time PCR is 0.0000005040504. Tm value obtained during melt curve analysis was also described in the **Appendix 2.**The standard curve shown in Figure 3.9 had slope of -3.945 and had efficiency of 95.042%. The T_M value (Figure) indicated that the same WSSV (82.1ºC) amplicon in the sample.

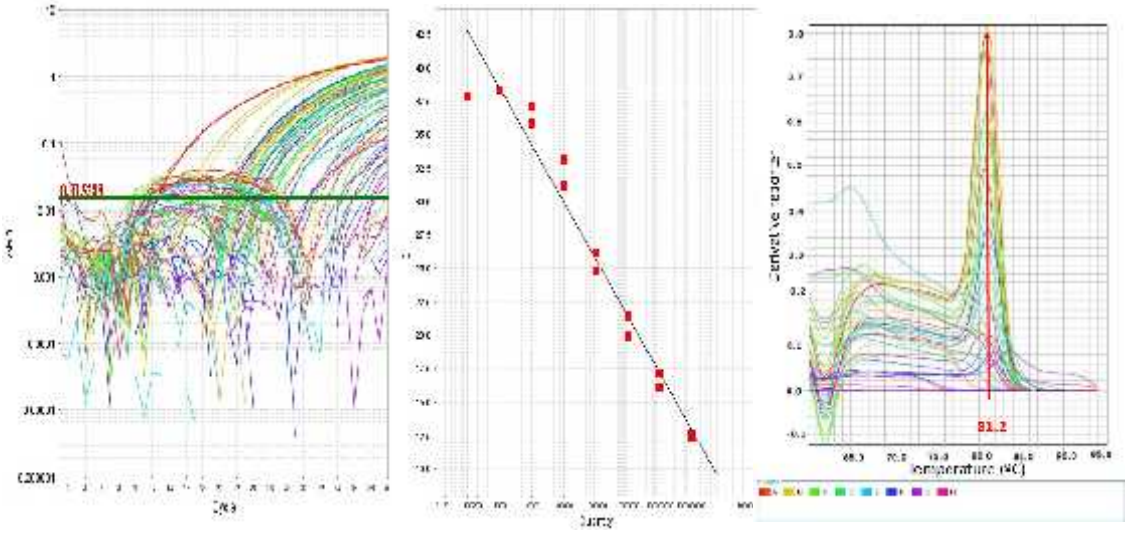


Figure 3.9 A) Amplification Plot B) Standard curve C) Melting Temperature

3.5.2 Overall seasonal change in viral load (copy number/g)

Of three seasons, IAM and DM had nearly 2453- and 16-times higher viral load than in the IBM ($3.52 \pm 0.22 \times 10^{8}$; **Figure 3.10**).

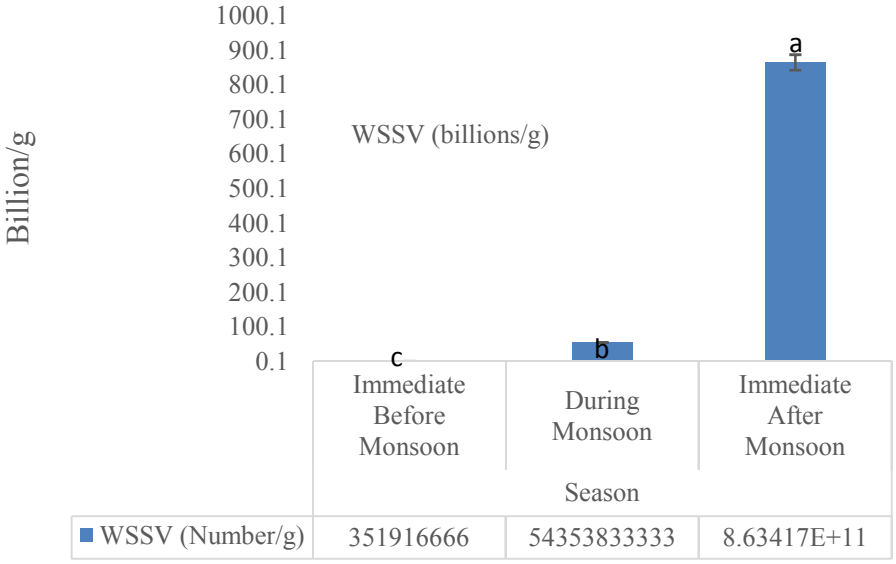


Figure 3.10 Overall viral load (Billions/g) across all three Seasons. Bars (mean ± 1 SEM) with different letters are significantly different (ANOVA, HSD; P<0.05).

3.5.3 Overall spatial change in viral load (copy number/g)

Of four sampling sites, Asasuni, Debhata and Tala had nearly 1.30-, 1.13- and 1.11-folds higher viral load than did Satkhira Sadar ($2.57 \pm 0.12 \times 10^{11}$; **Figure 3.11**).

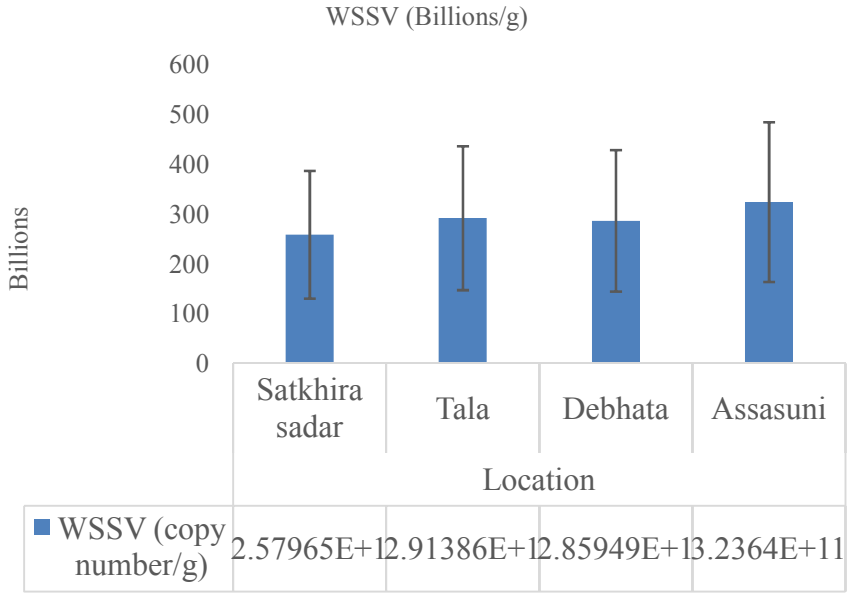


Figure 3.11 Overall viral load (Billion/g) across all four durations regardless of the seasons. Bars (mean ± 1 SEM) with no letters denote no significant difference (ANOVA, HSD; P<0.05).

3.5.4 Seasonal change in viral load (copy number/g) across sampling sites

In IBM the viral load in Satkhirasadar, Tala, Debhata and Assasuni was 2.40 ± 0.24 × 10^8,3.7 ± 0.15 × 10^8, 3.63 ± 0.117 × 10^8 and 4.34 ± 0.18 × 10^8, respectively (Figure 3.12).

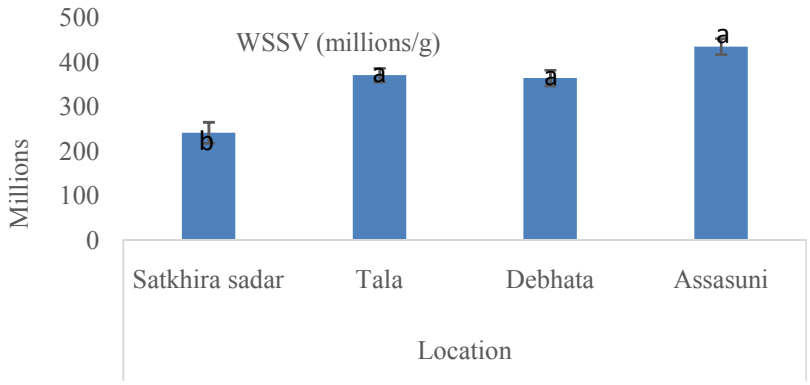


Figure 3. 12 In IBM, viral load (millions/g) across all four locations. Bars (mean ± 1 SEM) with different letters denote significant difference (ANOVA, HSD; P<0.05).

The viral abundance in DM season among four sampling sites i.e., Satkhirasadar, Tala, Debhata and Assasuni was 4.32 ± 0.12 × 10^9, 5.78 ± 0.07 × 10^9, 5.15 ± 0.02 × 10^9 and 6.49 ± 0.25 × 10^9, respectively **(Figure 3.13)**.

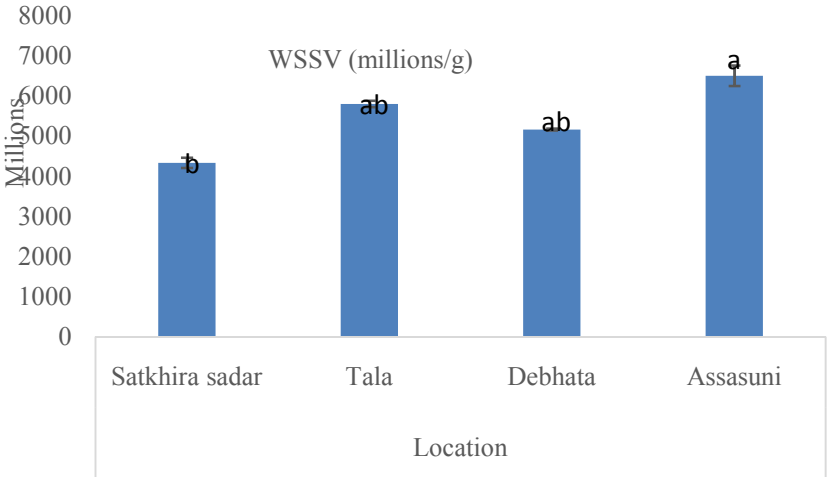


Figure 3.13 In season DM, viral load (millions/g) across all four locations. Bars (mean ± 1 SEM) with different letters indicate significant difference (ANOVA, HSD; P<0.05).

In IAM while Satkhirasadar had 7.69 ± 0.25 × 10^{11} viral density, Tala, Debhata and Assasuni had 8.68 ± 0.17 × 10^{11}, 8.52 ± 0.22 × 10^{11} and 9.64 ± 0.13 × 10^{11} viral abundance **(Figure 3.14)**.

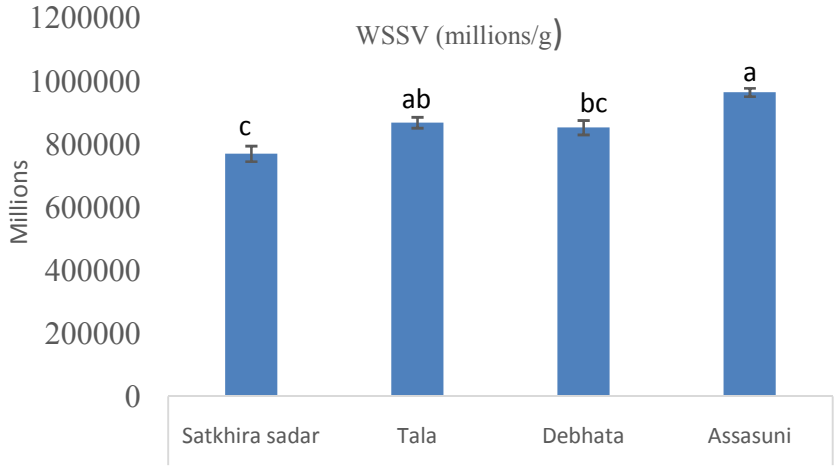


Figure 3.14 In IAM season, viral load (millions/g) across all four locations. Bars (mean ± 1 SEM) with different letters are significantly different (ANOVA, HSD; P<0.05).

3.6 Sensitivity Analysis of Conventional and Real-Time (SYBR green) PCR

The sensitivity of conventional PCR in this study found 50000 copy**(Figure 3.15)** whereas the SYBR green Real-Time PCR was 49 copy per Reaction **(Figure 3.16).**

Figure 3.15 Tenfold dilution series (left) which showed Conventional PCR can detect upto 10^5 copy/g. Twofold dilution series (right) which showed Conventional PCR can detect upto 50000 copy/g.

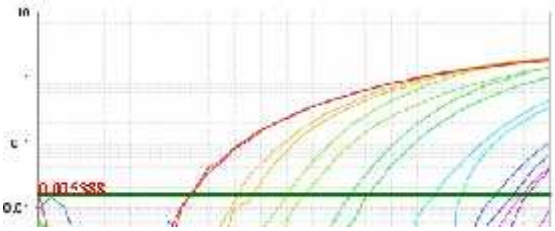


Figure 3.16 Real-Time amplification plot of two fold dilution series

3.7 Bioinformatics Study

3.7.1 Sequence Comparison

In the BLASTn feedback scenario the closest hit of BAN_135_S1_2015_VP28, BAN_241_S2_2015_VP28 and BAN_323_S3_2015_VP28**(Appendix 8)** was WSSV isolate BAN SH MU-23 2014 VP28 gene (KP21387), WSSV strain CN01 Complete genome (KT995472) and WSSV strain CN03 (KT995471). The identity of this hit was100%,100%, 99% and covering 100%, 100%, 97% of the query sequence, whereas the max score of the hit against query was1096,881, 1094 and Expect Value (E-value) of getting another hit of the database for the three isolates was zero in all the isolates.

3.7.2 Phylogenetics analysis

In the present study the BAN_135_S1_2015_VP28 isolates clustered with other Bangladeshi isolates found in other study. The Bangladeshi

isolatesBAN_241_S2_2015_VP28 and BAN_323_S3_2015_VP28 branched out as a leaf from which were found to be the closest hits of the WSSV isolate china 99/Qinado VP28 gene(GenBank Accession no AY249440)in BLASTn search. Hepatitis B virus surface protein was added as an out-group. Rooted phylogenetic tree was constructed using the neighbour-joining method. The robustness of the tree topology was assessed with 1000 bootstrap replicates **(Figure 3.17)**.

The strain BAN_135_S1_2015_VP28 is 99.81% similar (compairing with 516bp) with Bangladeshi strain WSSV BAN SH AL-1 VP28 gene (GenBank Accession no KJ817413). Only one difference (G in BAN_135_S1_2015_VP28 and A in BAN SH AL-1 VP28 gene) in position 516.

Compairing 516bp 100% similiarity between BAN_241_S2_2015_VP28 and WSSV isolate china 99/Qinado VP28 gene (GenBank Accession no AY249440). When compairing 597bp sequences with WSSV isolate china 99/Qinado VP28 gene (GenBank Accession no AY249440) it showed 99.16% similarity (change in 2, 3,105,596 and 597 bp).

In comparing BAN_323_S3_2015_VP28 with complete gene sequences of WSSV isolate china 99/Qinado VP28 gene (GenBank Accession no AY249440) it showed 92% similiarity (Change in position 45, 46, 148, 639 and 640).

3.7.3 Comparison of the Amino Acid Sequence with Reference Sequences

The alignment of amino acid shows substitution in position 10 and 135 with the chosen reference sequences **Figure 3.19**

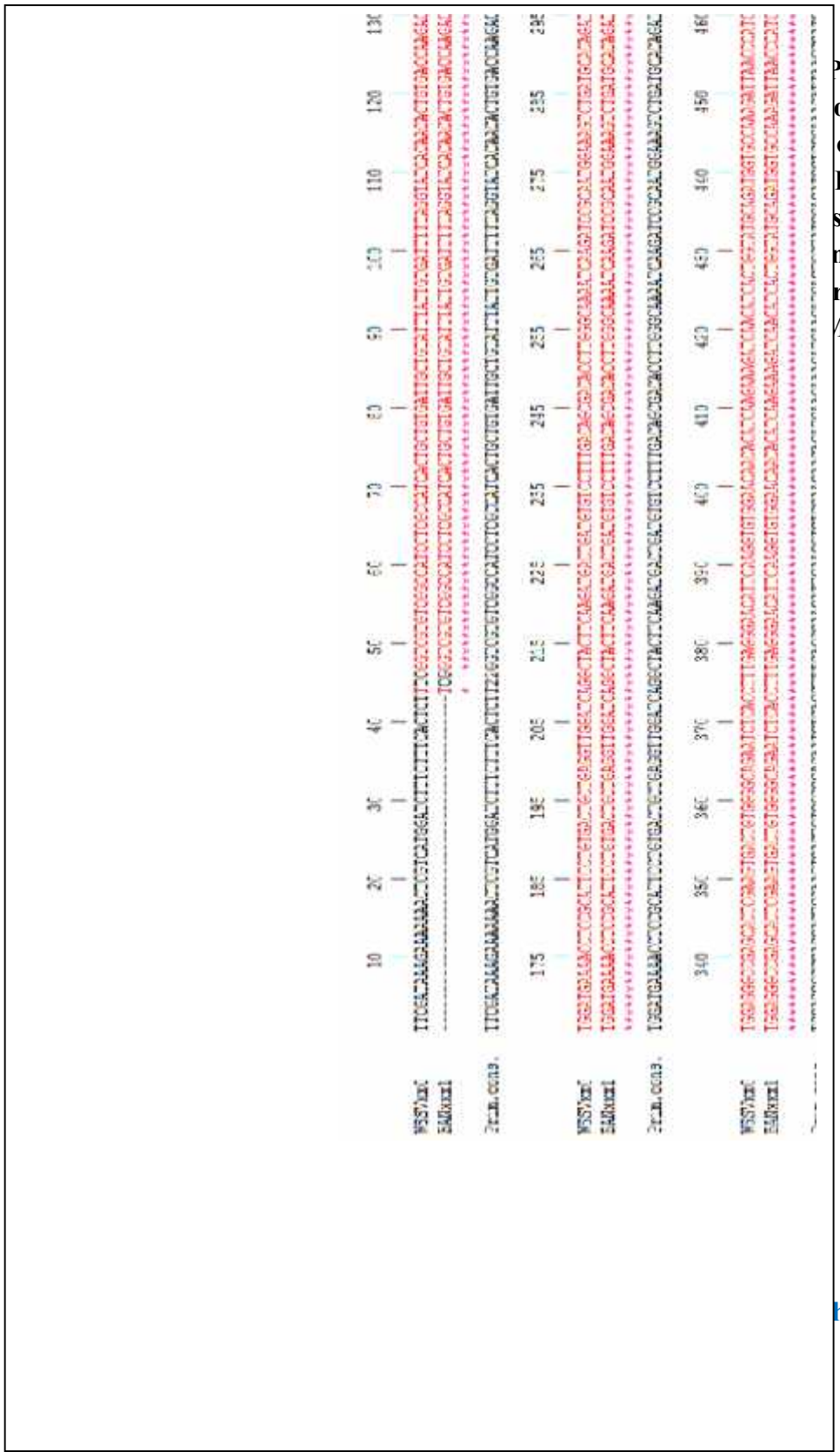


Figure 3.17 Phylogenetic tree constructed based on sequences of P28 gene genes of selected samples and corresponding references using MEGA6 (Tamura *et al*. 2013)

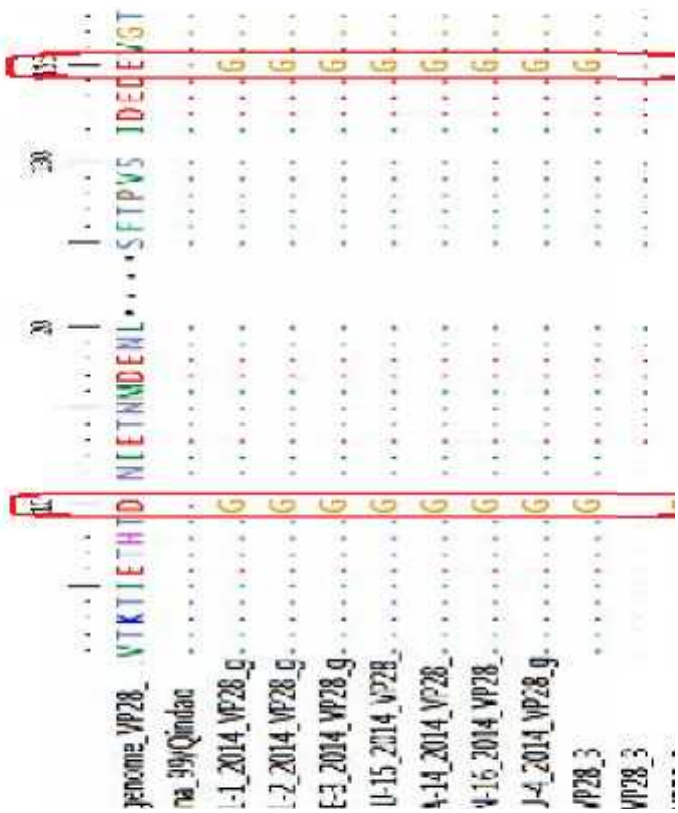


Figure 3.18 Comparison BAN_323_S3_2015_VP28 with complete gene sequences of WSSV isolate china 99/Qinado VP28 gene (Gen Bank Accession

Discussion

Chapter 4

VP19, VP28, WSV360, VP664, ORF191 and ORF36 are the main target for the detection of WSSV. Among this, VP28 (contains 204 amino acid residues with a theoretical molecular mass of 22 kDa) is the most abundant exposed protein in the WSSV envelope. It is thought that WSSV VP28 plays a critical role during early events of virus infection, particularly as a viral attachment protein. This protein has high homology to the recognition of receptors at the shrimp cell (Rab7) surface (Tang *et al*., 2007). There are plenty of sequences of VP28 gene available in GenBank, which contributes in designing high-coverage primers and probes. Thus, due to its essential role and the conserved nature of the gene encoding, VP28 among several WSSV geographical isolates, it seems as an appropriately sensitive target for PCR diagnosis in shrimp.

The PCR negative sample were tested to conform that if DNA was present or not. Sometimes the negative in PCR gave false result because during automated DNA extraction there will be some mechanical error which leads to no DNA in the extracted sample. Therefore, to conform whether DNA was present or not this step was done. From the results, it is clear that in all PCR negative sample contained DNA. Thus this test result is satisfactory.

An interesting observation of the present study is that some of the shrimp samples (25%, 10 out of 40 positive samples) did not show external spot or characteristics symptoms of WSSV although they were positive in PCR test. Similar findings has been supported by Hossain *et al*. (2014). Therefore, no characteristic symptoms indicates cultured shrimps without anycharacteristicexternal spots of WSD can act as a carrier of WSSV.

The present study depicted that the immediate after monsoon season is the most vulnerable season for cultured shrimp. During this time, the WSD prevalence across all four sampling sites were 93.75%. The lowest WSSV prevalence was found in immediate before monsoon season (75%). The moderate prevalence was detected in monsoon season (81.25%). This high rate of prevalence in monsoon season that has been justified as most likely being warmer temperatures, changing water current including heavy rainfall and pH which favors the multiplication and survival of WSSV in the shrimp

(Iqbal *et al*., 2011). Low salinity or rapid reduction in salinity is a predisposing unfavorable factor , where shrimps are stressed, may trigger the favorable condition for WSSV replication leading to WSD and impacts on shrimp aquaculture (Karim *et al*., 2012). Therefore, the occurrence of most WSD by WSSV as found in this study during immediate after monsoon season that could have resulted due to rapid fluctuation of salinity, temperature and pH, may trigger virus infection.

A minimum of five logs of WSSV copies/g of tissue sample was necessary to establish disease in shrimp (Durand and Lightner, 2002). In all location throughout the study season the average viral load was greater than five logs which denotes that a severe viral load persist throughout the seasons. It is interesting to note that WSSV seasonal prevalence correlated (proportional) with seasonal viral load.

The great fluctuation of salinity observed towards the coast than from the distance from the coast(Ruksana*et al*., 2013). An interesting findings is that towards the coast WSSV prevalence and viral load increases, which indicates the rapid fluctuation of salinity propagates viral load and prevalence. The similar result has been observed in the west coast of India (Chakrabarty*et al*., 2014).

In Satkhira most of the farm practice extensive culture system where scientific management is not applied. Traditionally PLare stocked and harvest from the gher. Tidal water is filled in and drained out by using the same structure as inlet and outlet of the ghers..Debnath*et al*. (2014) observed that exposure to extremes of single factor may be tolerated; however, combinations may be adverse or lethal. The higher prevalence suggests that poor pond management and improper measures to sudden changes in weather conditions such as temperature, salinity, pH could cause stress and subsequent disease outbreak.

Therefore, the observed seasonal variations of WSSV prevalence in cultured shrimp will provide the basis for awareness building at farm level and to develop strategies for proper farm management practices.

The sensitivity of Conventional and Real-Time PCR performed in this study have shown that the Real-Time PCR is nearly 1000 times more sensitive than conventional PCR (can

detect upto 50000 copy). Although all conventional PCR negative sample of this study showed negative in the Real-Time PCR. The main reason behind showing negative in conventional PCR could be because of low viral load although the same sample may contain WSSV whose density is lower than 50000. Therefore, the development of rapid and sensitive detection method using molecular tools will be helpful not only for the detection of WSSV in cultured shrimp but also for quarantine of brood shrimp in harvesting from wild or imported from abroad.

Phylogenetic analysis reveals that all VP28 gene sequence of local samples clustered together flanked by other VP28 reference sequences which indicates similar WSSV lineage circulates in Bangladesh. The reference sequences available in database shows a minimum level of variation of the local sequence of WSSV that is in agreement with the findings of Hossain *et al*. (2014).

From this observation it can be suggested that the cross border animal movement could be responsible for WSSV outbreak in Bangladesh. Lack of proper quarantine on import of fish and fish products from WSSV endemic country as well as legal or illegal cross border animal movement are the main obstacles for WSSV control in Bangladesh.

The isolates WSSV VP28 gene (BAN_135_S1_2015_VP28) show two substitution in amino acid sequence in position 10 (Aspartate instead of Glycine) and 135(Glutamate instead of Glycine) in respect to the reference sequence of WSSV isolate china 99/QinadoVP28 gene (GenBank Accession no AY249440). BAN_241_S2_2015_VP28 coincide with reference sequence of china.

The BAN_323_S3_2015_VP28 isolates shows a substitution Glycine at position 10 instead of Aspartate in the reference sequenceWSSV isolate china 99/Qinado VP28 gene (GenBank Accession no AY249440) and substitution of Glutamate insteadGlysine at position 135 in respect to previously reported Bangladeshi sequences (Hossain *et al*. 2014). This study finds this is a new mutation which is not yet reported. This implies at least three single genetic strains of WSSV has been circulating and the mutation rate is very low.

This outcome supports the view that commercial activities such as the long-range transport of brood stock and post larvae may have played an instrumental role in the spread of WSSV. One strain (BAN_135_S1_2015_VP28) coincide with the reference strain of China (GenBank Accession no AY249440) which denotes that the China strain somehow established in the study area. This may be due to the imports of huge brood shrimp and shrimp feed from China when wild brood shrimp in the spawning season was unavailable.

Islam *et al*. (2014)proposed that an organism adapts to its environment by the substitution of mutations that slightly enhance fitness, because mutations resulting in small fitness changes are more likely to be beneficial than mutations causing large fitness changes. Mutations leading to larger fitness enhancement had a larger probability of becoming established. However, the role of mutation as found in this study is still unknown and need further study.

Shrimp lack the adaptive immune response which provide a versatile mechanism for natural protection (Islam *et al*. 2014). Moreover, strategies for health management in shrimp are primarily based on exclusion of pathogen and the avoidance of environmental condition. Therefore, proper quarantine measures of imported brood and shrimp feed as well as environmental parameters controlling will enhanced the overall production of shrimp.

Conclusions and Recommendations

Chapter 5

5.1 Conclusions

Considering the results presented in this study this may be concluded that SYBR green technique can be used as a method to detect viral load which is 1000 times more sensitive than the conventional PCR technique.

Coastal waters are more favorable in the outbreak of WSD.

A new mutation in amino acid sequence (position in 135) that can be taken into consideration in designing vaccines if anybody is interested.

5.2 Recommendations

1. Need further study to develop technique to quantify viral presence when the load will be only one copy.

2. Need proper quarantine measures before releasing imported broods.

3. Adopt scientific semi-intensive management technique to prevent WSD outbreak.

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Appendices

Appendix 1

Details of the WSSV samples during sample collection and PCR profile of all samples for present study

Serial no	Sample ID	Upazilla	Season	PCR amplification
1	BAN 111	Satkhirasadar	Immediate before monsoon	Negative
2	BAN 112	Satkhirasadar	Immediate before monsoon	Positive
3	BAN 113	Satkhirasadar	Immediate before monsoon	Positive
4	BAN 114	Satkhirasadar	Immediate before monsoon	Positive
5	BAN 121	Tala	Immediate before monsoon	Positive
6	BAN 122	Tala	Immediate before monsoon	Positive
7	BAN 123	Tala	Immediate before monsoon	Negative
8	BAN 124	Tala	Immediate before monsoon	Positive
9	BAN 131	Debhata	Immediate before monsoon	Positive
10	BAN 132	Debhata	Immediate before monsoon	Negative
11	BAN 133	Debhata	Immediate before monsoon	Positive
12	BAN 135	Debhata	Immediate before monsoon	Positive
13	BAN 141	Assasuni	Immediate before monsoon	Positive
14	BAN 142	Assasuni	Immediate before monsoon	Positive
15	BAN 143	Assasuni	Immediate before monsoon	Positive
16	BAN 144	Assasuni	Immediate before monsoon	Negative
17	BAN 211	Satkhirasadar	During monsoon	Positive
18	BAN 212	Satkhirasadar	During monsoon	Positive
19	BAN 213	Satkhirasadar	During monsoon	Positive
20	BAN 214	Satkhirasadar	During monsoon	Negative
21	BAN 221	Tala	During monsoon	Positive
22	BAN 222	Tala	During monsoon	Positive
23	BAN 223	Tala	During monsoon	Positive
24	BAN 224	Tala	During monsoon	Negative
25	BAN 231	Debhata	During monsoon	Positive
26	BAN 232	Debhata	During monsoon	Positive
27	BAN 233	Debhata	During monsoon	Positive
28	BAN 234	Debhata	During monsoon	Negative
29	BAN 241	Assasuni	During monsoon	Positive
30	BAN 242	Assasuni	During monsoon	Positive
31	BAN 243	Assasuni	During monsoon	Positive
32	BAN 244	Assasuni	During monsoon	Positive
33	BAN 311	Satkhirasadar	Immediate after monsoon	Positive

34	BAN 312	Satkhirasadar	Immediate after monsoon	Negative
35	BAN 313	Satkhirasadar	Immediate after monsoon	Positive
36	BAN 314	Satkhirasadar	Immediate after monsoon	Positive
37	BAN 321	Tala	Immediate after monsoon	Positive
38	BAN 322	Tala	Immediate after monsoon	Positive
39	BAN 323	Tala	Immediate after monsoon	Positive
40	BAN 324	Tala	Immediate after monsoon	Positive
41	BAN 331	Debhata	Immediate after monsoon	Positive
42	BAN 332	Debhata	Immediate after monsoon	Positive
43	BAN 333	Debhata	Immediate after monsoon	Positive
44	BAN 334	Debhata	Immediate after monsoon	Positive
45	BAN 341	Assasuni	Immediate after monsoon	Positive
46	BAN 342	Assasuni	Immediate after monsoon	Positive
47	BAN 343	Assasuni	Immediate after monsoon	Positive
48	BAN 344	Assasuni	Immediate after monsoon	Positive

Appendix2

WSSV prevalence determined by conventional PCR among cultured *Penaeusmonodon*collected during the immediate before monsoon, monsoon and immediate after monsoon seasons from Satkhirasadar, Tala, Debhata, and Assasuni of Satkhira district.

Geographic Location	Season	Total no. of sample	WSSV positive	WSSV Negative	Prevalence %
SatkhiraSadar	**Immediately before Monsoon**	**4**	**3**	**1**	**75**
	During Monsoon	**4**	**3**	**1**	**75**
	Immediately after Monsoon	**4**	**3**	**1**	**75**
Tala	**Immediately before Monsoon**	**4**	**3**	**1**	**75**
	During Monsoon	**4**	**3**	**1**	**75**
	Immediately after Monsoon	**4**	**4**	**0**	**100**
Debhata	**Immediately before Monsoon**	**4**	**3**	**1**	**75**
	During Monsoon	**4**	**3**	**1**	**75**
	Immediately after	**4**	**4**	**0**	**100**

	Monsoon				
Assasuni	**Immediately before Monsoon**	**4**	**3**	**1**	**75**
	During Monsoon	**4**	**4**	**0**	**100**
	Immediately after Monsoon	**4**	**4**	**0**	**100**

Appendix3

Standard dilution series, quantity of DNA, C_T mean and melting temperature (Tm) of recombinant plasmid DNA based standard curve.

Standard dilution of VP28 gene	**Quantity of DNA (ng)**	**C_T mean± s.d.**	**Tm**
10^9	5.040504	12.527 ± 0.139	80.673
10^8	0.5040504	16.592 ± 0.747	80.866
10^7	0.05040504	20.665 ± 1.024	81.059
10^6	0.005040504	25.528 ± 0.957	80.866
10^5	0.0005040504	32.167 ± 1.419	81.446
10^4	0.00005040504	36.484 ± 0.899	81.446
10^3	0.000005040504	38.25± 0.012	81.253
10^2	0.0000005040504	38.824± 0.124	80.866

Appendix 4

Viral load calculation

Mean and SEM of the WSSV copy						
						WSSV
season	IBM	Location	Satkhira sadar	IBM		211000000.00
				DM		287000000.00
				IAM		223000000.00
				Total	Mean	240333333.3333
					SEM	23589074.68394
			Tala	IBM		340000000.00
				DM		391000000.00
				IAM		379000000.00
				Total	Mean	370000000.0000
					SEM	15394804.31834
			Debhata	IBM		390000000.00
				DM		330000000.00
				IAM		370000000.00
				Total	Mean	363333333.3333
					SEM	17638342.07376
			Assasuni	IBM		470000000.00
				DM		419000000.00
				IAM		413000000.00
				Total	Mean	434000000.0000
					SEM	18083141.32003
			Total	Mean		351916666.6667
				SEM		22617859.10525
	DM	Location	Satkhira sadar	IBM		4090000000.00
				DM		4340000000.00
				IAM		4530000000.00
				Total	Mean	4320000000.0000
					SEM	127410099.02411
			Tala	IBM		5890000000.00
				DM		5630000000.00
				IAM		5840000000.00
				Total	Mean	5786666666.6667
					SEM	79652020.96899

Continued Appendix 4 **Mean and SEM of the WSSV copy**

			Debhata	IBM		5140000000.00
				DM		5120000000.00
				IAM		5190000000.00
				Total	Mean	5150000000.0000
					SEM	20816659.99466
			Assasuni	IBM		6560000000.00
				DM		6010000000.00
				IAM		6890000000.00
				Total	Mean	6486666666.6667
					SEM	256666666.66667
			Total	Mean		5435833333.3333
				SEM		249175787.81319
			Satkhira sadar	IBM		797000000000.00
				DM		720000000000.00
				IAM		791000000000.00
				Total	Mean	769333333333.3334
					SEM	24727402703.16404
			Tala	IBM		881000000000.00
				DM		834000000000.00
				IAM		889000000000.00
				Total	Mean	868000000000.0000
					SEM	17156145643.27703
	IAM	Location	Debhata	IBM		812000000000.00
				DM		854000000000.00
				IAM		891000000000.00
				Total	Mean	852333333333.3334
					SEM	22820556035.68366
			Assasuni	IBM		987000000000.00
				DM		963000000000.00
				IAM		942000000000.00
				Total	Mean	964000000000.0000
					SEM	13000000000.00000
			Total	Mean		863416666666.6667
				SEM		22514459439.17783
	Total	Mean				289734805555.5555
		SEM				68955184761.72421

Report

WSSV

season	Mean	Std. Error of Mean
IBM	351916666.6667	22617859.10525
DM	5435833333.3333	249175787.81319
IAM	863416666666.6667	22514459439.17783
Total	289734805555.5555	68955184761.72421

Report

WSSV

Location	Mean	Std. Error of Mean
SS	257964555555.5555	128042682440.49966
T	291385555555.5555	144240781952.92750
D	285948888888.8889	141750958476.52087
A	323640222222.2222	160136324383.56128
Total	289734805555.5555	68955184761.72421

Tests of Between-Subjects Effects

Dependent Variable: LNWSSV

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	379.373[a]	11	34.488	6927.569	.000
Intercept	19341.900	1	19341.900	3885139.702	.000
season	378.436	2	189.218	38007.573	.000
Location	.795	3	.265	53.235	.000
season * Location	.141	6	.024	4.735	.003
Error	.119	24	.005		
Total	19721.392	36			
Corrected Total	379.492	35			

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

LNWSSV

	Location	N	Subset for alpha = 0.05	
			1	2
TukeyHSD[a]	SS	3	19.2883	
	T	3		19.7084
	D	3		19.7272
	A	3		19.8869

LNWSSV					
	season	N	Subset		
			1	2	3
TukeyHSD[a,b]	IBM	12	19.6527		
	DM	12		22.4045	
	IAM	12			27.4804
	Sig.		1.000	1.000	1.000
Means for groups in homogeneous subsets are displayed. Based on observed means. The error term is Mean Square(Error) = .005.					
a. Uses Harmonic Mean Sample Size = 12.000.					
b. Alpha = .05.					

Onewayanova in IBM season in four locations

LNWSSV

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.589	3	.196	17.605	.001
Within Groups	.089	8	.011		
Total	.678	11			

	Sig.		1.000	.241

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

1-way ANOVA for WSSV loads in DM in four locations

LNWSSV

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.270	3	.090	44.789	.000
Within Groups	.016	8	.002		
Total	.286	11			

LNWSSV

	Location	N	Subset for alpha = 0.05		
			1	2	3
TukeyHSD[a]	SS	3	22.1856		
	T	3		22.3622	
	D	3		22.4786	22.4786
	A	3			22.5914
	Sig.		1.000	.052	.059

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

1-way ANOVA in IAM in four locations

LNWSSV

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.077	3	.026	14.534	.001
Within Groups	.014	8	.002		
Total	.092	11			

LNWSSV

	Location	N	Subset for alpha = 0.05		
			1	2	3
TukeyHSD[a]	SS	3	27.3677		
	T	3	27.4705	27.4705	

	D	3		27.4891	27.4891
	A	3			27.5942
	Sig.		.068	.947	.062

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 5

Unless otherwise mentioned, all media were sterilized by autoclaving at 121° C for 15 minutes at 15 lbs pressure. Double distilled water was used for preparation of all media. The media used in this thesis have been given below:

1. **Nutrient Agar (OXOID)**

Ingredients	Amount (g/L)
Peptone	5.0
Sodium Chloride	5.0
Beef extract	3.0
Agar	15.0
pH	7.0

2. **Luria Bertani Agar (ROTH)**

Ingredients	Amount (g/L)
Trypton	10
Yeast Extract	5
NaCl	10
Agar	15
pH	7.0

3. **Luria Bertani Broth (ROTH)**

Ingredients	Amount (g/L)
Trypton	10
Yeast Extract	5

NaCl	10
pH	7.0

4. S.O.C Medium

Ingredients	Amount (g/L)
2% Tryptone	20
0.5% Yeast Extract	5
5 M NaCl	2
1 M KCl	2.5
1 M $MgCl_2$	10
1 M $MgSO_4$	10
1 M Glucose	20
Double distilled H_2O	Upto 1000 ml

5. LB Agar with 50 µg/ml Kanamycin

Ingredients	Amount (g/L)
Trypton	10
Yeast Extract	5
NaCl	10
Agar	15
pH	7.0
Kanamycin (100mg/ml)	500 µl (added after autoclave)

6. LB Broth with 100 µg/ml Kanamycin

Ingredients	Amount (g/L)
Trypton	10
Yeast Extract	5
NaCl	10

pH	7.0
Kanamycin (100mg/ml)	1 ml (added after autoclave)

7. X.L.D Agar

Typical Formula	gm/litre
Yeast extract	3.0
L-Lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium desoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	12.5
pH 7.4 ± 0.2 @ 25°C	

Appendix6

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 $MgCl_2$

20.33 g of $MgCl_2$ 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 $MgSO_4$

24.648 g of $MgSO_4$ 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 $Na_2EDTA.2H_2O$ 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 Na_2 $(CH_3COOH).H_2O$ 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	Up to 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

ATP^{TM} 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

Appendix 7

Instruments & Apparatus

The important instrument and apparatus used through the study are listed below:

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, Model no: MP220	Eppendorf, Germany

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
Maxwell^{R} 16 Instrument	Promega, USA
Real-time PCR system 7500	Applied Biosystems, Foster City, CA, USA
Dry block Heating/Cooling thermostat	Grant – bio, England

Appendix 8

>BAN 135 S1 2015 VP28

CGGTCGTGTCGGCCATCCTCGCCATCACTGCTGTGATTGCTGTATTTATTGTGATTTTTA
GGTATCACAACACTGTGACCAAGACCATCGAAACCCACACAGGCAATATCGAGACAAAC
ATGGATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGCTACTTCAAGAT
GACTGATGTGTCCTTTGACAGCGACACCTTGGGCAAAATCAAGATCCGCAATGGAAAGT
CTGATGCACAGATGAAGGAAGAAGATGCGGATCTTGTCATCACTCCCGTGGAGGGCCG
AGCACTCGAAGTGACTGTGGGGCAGAATCTCACCTTTGAGGGAACATTCAAGGTGTGGA
ACAACACATCAAGAAAGATCAACATCACTGGTATGCAGATGGTGCCAAAGATTAACCCA
TCAAAGGCCTTTGTCGGTAGCTCCAACACCTCCTCCTTCACCCCCGTCTCTATTGATGAG
GATGGAGTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTACCGC
CGGTGGAAATCTTTTCGACATGTACGTGCACGTCACCTACTCTGGCACTGAGACCGAGT
AA

>BAN 241 S2 2015 VP28

TCGAGACAAACATGGATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGC
TACTTCAAGATGACTGATGTGTCCTTTGACAGCGACACCTTGGGCAAAATCAAGATCCG
CAATGGAAAGTCTGATGCACAGATGAAGGAAGAAGATGCGGATCTTGTCATCACTCCCG
TGGAGGGCCGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACCTTTGAGGGAACATT
CAAGGTGTGGAACAACACATCAAGAAAGATCAACATCACTGGTATGCAGATGGTGCCAA
AGATTAACCCATCAAAGGCCTTTGTCGGTAGCTCCAACACCTCCTCCTTCACCCCCGTCT
CTATTGATGAGGATGAAGTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATT
GCAGCTACCGCCGGTGGAAATCTTTTCGACATGTACGTGCACGTCACCTACTCTGGCAC
TGAGA

>BAN 323 S3 2015 VP28

**TCGGGTCGTGTCGGCCATCCTCGCCATCACTGCTGTGATTGCTGTATTTATTGTGATTTT
TAGGTATCACAACACTGTGACCAAGACCATCGAAACCCACACAGGCAATATCGAGACAA
ACATGGATGAAAACCTCCGCATTCCTGTGACTGCTGAGGTTGGATCAGGCTACTTCAAG
ATGACTGATGTGTCCTTTGACAGCGACACCTTGGGCAAAATCAAGATCCGCAATGGAAA
GTCTGATGCACAGATGAAGGAAGAAGATGCGGATCTTGTCATCACTCCCGTGGAGGGC
CGAGCACTCGAAGTGACTGTGGGGCAGAATCTCACCTTTGAGGGAACATTCAAGGTGTG
GAACAACACATCAAGAAAGATCAACATCACTGGTATGCAGATGGTGCCAAAGATTAACC
CATCAAAGGCCTTTGTCGGTAGCTCCAACACCTCCTCCTTCACCCCCGTCTCTATTGATG
AGGATAAGTTGGCACCTTTGTGTGTGGTACCACCTTTGGCGCACCAATTGCAGCTACCG
CCGGTGGAAATCTTTTCGACATGTACGTGCACGTCACCTACTCTGGCACTGAGACCGAG
TAAGA**