INCIDENCE AND SEVERITY OF MAJOR POTATO VIRUSES IN SELECTED NORTHERN REGIONS OF BANGLADESH AND MOLECULAR DETECTION THROUGH RT-PCR

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*This is to certify that the thesis entitled, "**INCIDENCE AND SEVERITY OF MAJOR POTATO VIRUSES IN SELECTED NORTHERN REGIONS OF BANGLADESH AND MOLECULAR DETECTION THROUGH RT-PCR**" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN, PLANT PATHOLOGY, embodies the result of a piece of bona fide research work carried out by **MST. SHAHANA SULTANA,** bearing **Registration No. 13-05408** under my supervision and my guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

Dated:
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Dedicated to…

My beloved parents and the farmers who feed the nation

ABBREVIATIONS AND ACRONYMS

bp	**Base pair**
cDNA	**Complementary DNA**
CIP	**International Potato Center**
CMV	**Cucumber mosaic virus**
DNTPs	**Deoxyribonucleotide triphosphates**
dsRNA	**Double stranded RNA**
DTT	**Dithiothreitol**
EDTA	**Ethyline di-amine tetra-acetic acid**
ELISA	**Enzyme linked immune sorbent assay**
MEGA	**Molecular Evolutionary Genetics Analysis**
Mm	**Mili mole**
M-MLV	***Moloney murine leukemia virus***
mRNA	**Messenger RNA**
PCR	**Polymerase Chain Reaction**
PLRV	***Potato Leaf Roll Virus***
PLRV-CP	***PLRV*-Coat Protein**
PPV	***Plum pox virus***
PVA	***Potato virus A***
PVS	***Potato virus S***
PVV	***Potato virus V***
PVX	***Potato virus X***
RT-PCR	**Reverse Transcription Polymerase Chain Reaction**
ssRNA	**Single stranded ribonucleic acid**
rpm	**Rotation per minute**
TBE	**Tris Boric EDTA**
Taq	***Thermus aquaticus***
TE	**Tris EDTA**

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The Author

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ABSTRACT

In the survey study, incidence and severity of major potato viruses were demonstrated in the selected northern regions of Bangladesh. A lab experiment was also conducted for molecular detection of *PLRV*. The lab experiment was conducted in Molecular Biology and Plant Virology Laboratory under the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207 during January-February 2020. The survey study was conducted in three selected districts of the northern region viz. Bogura, Jaipurhat and Gaibandha. During the survey study, diseased samples were collected randomly and non-randomly. In total 270 leaf samples of potato were collected from the selected districts. Among the diseased sample 131 samples were infected with virus and virus like symptoms where *PLRV*+ve 72 samples, *PVY*+ve 52 samples and *PVX*+ve 7 samples. From the disease incidence and severity study on the basis of visual observation, it was revealed that *PLRV* was the most observed, predominant and devastating potato virus in all the selected districts and *PVY* also came in concern. Bogura was found to be the most infected region with *PLRV* (disease incidence 30.67% and severity 25.67%). For molecular detection of *PLRV,* in total 27 samples were considered from each spot and total RNA was extracted from the samples. Total RNA was then being subjected to cDNA synthesis. *PLRV* was confirmed through RT-PCR at 346bp fragments. Farmers were interested to grow the hybrid varieties for better production in the selected districts. BADC, cold storage and home storage were used as a source of seed tuber. From the study, it may be concluded that *PLRV, PVY* and *PVX* are found in all the selected districts. Among the identified potato viruses *PLRV* was common and appeared with severe outbreak. For detection of *PLRV,* RT-PCR was the most reliable, highly specific and robust molecular technique.

CHAPTER I

INTRODUCTION

INTRODUCTION

Potato (*Solanum tuberosum* L.) which belongs to the family *Solanaceae*, is the world's leading food and vegetable crop that originated in the Andean region of Peru. Globally, potato ranks the 4th largest staple crop after rice, wheat and maize in the world (Rauscher *et al.*, 2006; Islam *et al*., 2014 and Abbas *et al*., 2014). The potato crop contributes 12-15 times more yield per production per unit area that is higher than wheat, maize and rice. The potato tuber provides an excellent source of carbohydrates, protein and vitamins **(**Salazar *et al*., 2003 and Chiunga *et al.*, 2013). In the world, potato is cultivated 19,098,300.00 ha of lands and production is 381,682,000.00 tons (FAOSTAT 2017) and in Bangladesh, potato is cultivated is in 4, 68,375 ha of lands and production is 96, 55,082 metric tons (BBS 2018-19). In Bangladesh, potato is cultivated in 23 major growing areas. Comparing to other agricultural crops, cost of potato cultivation through seed tubers is much higher. Bangladesh Agricultural Development Corporation (BADC) reported that the cost of potato seed tuber is liable to 30 - 40% of total production cost (Anon *et al*., 2005). Bangladesh has imported high yielding foreign potato varieties which costs of above US $ 150.00 per quintal which engross large amount of foreign currency every year.

Potatoes affected by many abiotic and biotic factors. Abiotic factors such as hail, floods and droughts, and temperature-related events such as frost and heat waves etc. are causes 25% yield losses. High yielding foreign potato varieties significantly increased the yield of potato crop in our country but at the same time resulted new viral problems like *PLRV, PVY and PVX* which have been reported in Bangladesh and causes 10-90% yield losses. *Potato leaf roll virus (PLRV*) and *Potato virus Y (PVY)* are among the most damaging potato viruses and are prevalent in most potato growing areas. Viral diseases constitute a major constraint to high yield and high quality production of potato (Waterworth and Hadidi 1998). *Potato leaf roll virus* (*PLRV)* is the most damaging potato viruses and is prevalent in the most potato growing areas. Experimental data showed that

plants from *PLRV*-infected seed tubers produced at least 60% less total yield and 88% less marketable yield (tubers >85 g) than plants from healthy seed tubers. More than 35 different viruses are known to affect potatoes. Currently, no reports are available about high yielding commercial varieties or advance potato lines in Bangladesh has shown durable resistance against these viruses (Karim *et al.*, 2010).

Most viruses can effectively be determined by ELISA tests, but the serological methods can be unreliable for the detection all potato viruses like *PLRV*, because this virus often occurs at low concentration in plant tissue and virions are weakly immunogenic (Beemster *et al*., 1987). Reverse Transcription Polymerase Chain Reaction (RT-PCR) offers a potentially more sensitive method for detection of viruses from plant tissue and even from dormant tubers.

Viral diseases can often be diagnosed by color deviations (mosaic patterns) on leaves, leaf and stem rolling, growth reduction (stunting/dwarf) of the plant, malformations of plants and tuber net necrosis. Symptoms are not always visible sign attributable to interactions amid the virus and the potato plant. Growing factors, like weather and fertility, or stage of the plant when it is infected, also effects the expression of symptoms. Nucleic acid detection and serology apprehension techniques usually acclimatize to analyze and characterize suspected viral diseases. Molecular tests, like RT-PCR and serological tests (cocktail and sandwich ELISA) are used to diagnose *PLRV*. Saiki *et al.,* 1985 described that successful PCR reaction is because of the choice, quality & accuracy of various factors, like template DNA/cDNA (RT-PCR), primers, dNTPs, concentration of magnesium ion, choice of polymerase enzyme and primer's annealing temperature. As a first step of this study to develop RT-PCR mediated commercial scale screening protocol of *PLRV*. As RT-PCR gives higher sensitivity with high speed diagnosis and reduced sample size, therefore, it was found good alternative to other diagnostic methods like ELISA, etc. PCR assays for *PLRV* detection were made quite easy and possible by the database of

nucleotide sequences for many plant pathogens like viroids, viruses, etc. The RT-Similarly, a number of scientists have reported the usefulness of PCR for the detection of many plant viruses e.g. apple scar skin, grapevine virus A, pome fruit virus, and potato virus A from dormant tubers (Singh and Singh, 1998). This study was aimed to establish the status of viral disease in selected potato growing areas through the estimation of disease incidence, severity through molecular detection with the following objectives-

- To establish the status of major potato viruses in selected districts of Bangladesh
- To determine the disease incidence and severity in major potato viruses of the selected surveyed areas
- To optimize the reliable RT-PCR based molecular detection method for local strain of potato virus

CHAPTER II

REVIEW OF LITERATURE

REVIEW OF LITERTURE

2.1 Potato

Potato (*Solanum tuberosum*) is an herbaceous annual plant that belongs to the Solanaceae or "nightshade" family of flowering plants. It was originated in the Andes Mountains of South America more than 8000 years ago. The global total crop production of potato exceeds 376 million metric tons (Beuch *et al.,* 2013). It currently ranks the world's fourth most important food crop after maize, wheat, rice, and the first among root and tuber crops (Salazar *et al*., 2003 and Chiunga *et al*., 2013). More than 6 billion people worldwide eat potato, which is produced in over 130 countries worldwide (Salazar *et al*., 2003 and Beuch *et al*., 2013). Potato virus are widely distributed virus in potato all over the world as ranks the fifth among the top ten of the most economically damaging plant viruses worldwide.

2.2 Potato Infecting Viruses

Potato is susceptible to a wide range of pathogens including bacteria, fungi, nematodes, viruses and viroid's (Mulder *et al*., 2005). It is known to be infected by 175 diseases in addition to several physiological disorders (Islam *et al*., 2013). Viruses beside *Phytophthora infestance* (the causative agent of late blight) are the most important ones among potato pathogens (Salazar *et al*., 1996 and Mulder *et al*., 2005). Yield reduction of potato is attributed by many of these diseases, for example, the natural infection with *Potato virus Y* could cause a yield reduction up to 80% depending on the virus strain and potato cultivars. Apart from late blight, viruses are the most demonstrable seed-borne agents that affect vigor, yield and tuber quality of potato (Salazar *et al*. 1996 and Wale *et al*., 2008). More than 40 viruses naturally infect cultivated potato (Table 1). Some of these viruses notably *Potato leaf roll virus (PLRV), Potato virus Y (PVY), Potato virus A (PVA), Potato virus X (PVX), Potato virus M (PVM), Potato virus S (PVS)* and *Potato aucubar mosaic virus* (*PAMV)* occur worldwide in potato crops, while others are important only in some

geographical areas (Altaleb *et al*., 2011; Salazar *et al*., 1996; Khan *et al*., 2013 and Abbas *et al*., 2014). In order to spread successfully, Potato viruses need other agent to transfer from infected plants into other healthy ones, in a process called transmission. The transmission of any plant virus needs a cooperation of the virus, the transmitting agent, the plant and the environment under optimum conditions to achieve maximum efficiency (Wale *et al*., 2008). Potato viruses are naturally transmitted via three ways. The first way is through the parts of plants that used for propagation. Vegetative propagation of infected tubers is considered as the main source of virus infection through which the viruses pass from one generation to the next.

Insects as vectors are the second way for virus transmission; these could be mites, aphids, nematodes or fungi. Among the insect vectors of potato viruses aphids are the most important ones, because the two most damaging viruses in the crop, *PVY* and *PLRV*, are transmitted via aphid species. Over 50 aphid species are known to transmit potato viruses; they transmit the largest number of viruses. Another way for virus transmission is mechanical transmission, by contact between infected and healthy plant parts, including contact with contaminated farm machinery, operator hands, or animals. The viruses which are most frequently encountered in potato fields such as *PVY*, *PVX*, *PVS*, *PVA*, *CMV*, *PVV* and *TMV*, are mechanically transmitted with the exception of PLRV which is transmitted only by insect vector (Altaleb *et al*., 2011). Virus infections cause plant disease by affecting their metabolism. In virology, plant called primary diseased when they become infected during cultivation, while for plant that originated from infected tubers are secondary diseased plants. Plants with primary and secondary infection with the same virus usually shows the different symptoms (Salazar *et al*., 1996). Viruses affect the potato plants in different ways; they cause rapid degeneration of potato tubers, foliar malformation, and mild or sever mosaic, stunted growth and wilting. The severity of viruses' symptoms on potato depends on many factors including, potato varieties, virus strain, time of infection, age of crop and environmental

conditions (Wale *et al*., 2008 and Mulder *et al*., 2005). Indeed most symptoms of potato viruses are masked at high temperatures, this makes indexing of virus by visual observation very difficult, and many plants will look healthy while they are actually infected. Thus, in order to manage virus diseases and control their spread, reliable virus detection methods are needed.

Table 1. Categorization of importance of potato viruses based in three characteristics (Modified from Salazar *et al*., 1996)

Category	Viruses	Effect on yield	Risk analysis	Distribution
1	*PLRV, PVA, PVX*	Up to 90%	1	1
2	*APMoV, PVX, PAMV, PVV, PMTV, TRV, PYVV*	Up to 40%	1	2
3	*AMV, PVY, PBRV, TBRV, PVP, PVS, PRRDV*	Up to 20%	1-2	2
4	*PVT, APLV, PVU, WPMV, EMDV, TNV, TRSV, AVB(O)*	Up to 10%	1-3	2
5	*SALCV, ToSWV, APLV, PYDV, TSV, BCTV, CMV, PYMV*	Up to 10%	2-3	2

According to the table viruses in categories 1, 2 and some in category 3 can be considered the most economically important in potato production worldwide.

2.3 Symptomatology

2.3.1 *Potato leaf roll virus*

Symptoms: Foliar symptoms of *PLRV* include leaf rolling, chlorosis (yellowing), reddening, 'leathering' of leaves, phloem necrosis, and stunting (Jutta *et al*., 2009). Plants infected early in the growing season may also be dwarfed, but if virus infection occurs late in the growing season foliar symptoms may not be exhibited. Potato plants develop resistance to foliar infection with plant age (Erik *et al*., 1993*).* Many times, infection can be seen in a circular pattern in the field, and frequently surrounding what was most likely the original source of virus inoculums, an infected seed piece. Direct damage can also result from aphid feeding. Large numbers of aphids present can kill potato plants producing what are referred to as an 'aphid hole' in the field (Robert *et al*., 2000*). PLRV* travels through the phloem of the plant into tubers, reducing size and causing net necrosis. Net necrosis causes browning of the vascular system extending throughout the entire tuber. Tubers with net necrosis are unacceptable for processing into chips and fries, causing serious losses to growers and potato processors (Plate 1).

i) General Characteristics of *PLRV*

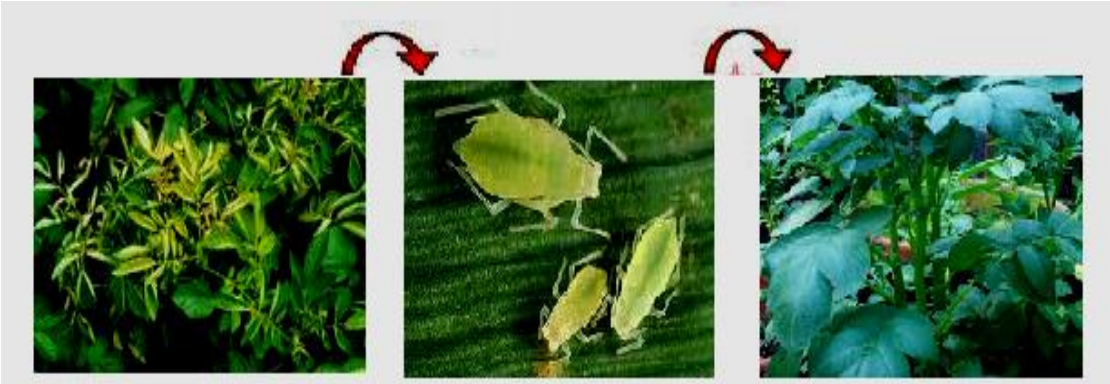
PLRV is under the Polerovirus genus and of the Luteoviridae family (Mayo and D'Arcy, 1999). Viruses under the Luteoviridae family have some unique characteristics. Viruses under this family are not mechanically transmitted. They are transmitted by aphids in a circulative way, which is obligatory transmission and its manner is non-propagative and viral infection is restricted mainly to phloem. This means, viruses under this family are not mechanically transmitted (Harrison, 1999). PLRV is an RNA type virus that infects potatoes worldwide and causing significant yield loss (Robert and Lemaire, 1999). It has been documented as one of the most damaging disease of potato crops in Pakistan as well. It is a persistent virus and continually presents major problems in leaf rolling and adversely affects quality seed tuber production (Gul *et al*., 2011).

a) Infected plant **b) Infected tuber**

Plate 1. Foliar symptoms of *PLRV* include leaf rolling, chlorosis (yellowing), 'leathering' of leaves, stunting and infected tuber. a) Infected plant b) Infected tuber.

ii) Disease Cycle of *PLRV*

All viruses are proved to be tuber borne. The viruses' transmission is automatic, from seed to seed or from plant section to plant section. *PLRV* is also persistently transmitted by aphids. A model transmission of *PLRV* by aphids is given below (Plate 2).



a) *PLRV* infected potato plants b) Viruleferous aphids c) Healthy potato plants

Plate 2. *PLRV* transmission from infected potato plants to healthy potato plants by Aphid vector. a) *PLRV* infected potato plants b) Viruleferous aphids c) Healthy potato plants.

2.3.2 *Potato virus X (PVX)*

Symptoms

i. Chlorotic streaks on leaves which become necrotic

ii. Inter veinal necrosis is appeared on leaves, petiole and stem

iii. Plant remain stunted in growth

iv. Twisting of the leaves occurs in combination with *PVX* and *PVY* (Figure 1)

Figure 1: *PVX* showing stunted in growth and leaf necrosis

2.3.3 *Potato virus Y (PVY)*

Symptoms

i. Sometime referred as latent potato mosaic

ii. Light yellow mottling with slight crinkling on potato plants

iii. Inter veinal necrosis of top foliage are showed

iv. Stunting of diseases plant

v. Leaves may appear slightly twisting where strains of *PVY* combines (Figure 2)

Figure 2: *PVY* showing inter veinal necrosis and light yellow mottling

2.4 Detection Methods of Plant Viruses

Viruses infect a wide range of plant species leading to cause many serious plant diseases in the world. The effect of viral diseases on plants quality and quantity leads to the loss of billions of dollars per year (Thesh *et al*., 2006 and Vender vlugt *et al*., 2006). Unfortunately, there are no chemical agents like bactericides and fungicides that can use against plant viruses (Fkhabad *et al*., 2012). Viral diagnosis is considered as one of the most valuable strategies for virus disease management. Thus to achieve an effective control, viruses must be accurately identified as an initial step of management. Diagnostic and detection techniques for plant viruses categorize based on: biological properties of the virus interaction with its host or vector, such as symptomology and transmission tests and intrinsic properties of the virus itself, like serological and nucleic acid-based techniques (Khabad *et al*., 2012 and Vender vlugt *et al*., 2006).

2.4.1 Diagnostic Methods Based on Biological Properties

i. Symptomatology

Symptoms on plant usually used if they are characteristic of a certain disease. Symptomatology relies on visual inspection of the symptoms expressed by viruses in plant. Plant viruses cause many symptoms on plant like mosaic, mottling, stunting, leaves malformation, necrosis, chlorosis, yield reduction or a combination of these symptoms. However, many biotic and a biotic factors could affect the appearance of these symptoms. Moreover, some plants could exhibit virus-like symptoms as a result of unfavorable conditions. Although symptoms provide essential information about virus infection, we can't make a decision on symptomatology alone, as some viruses cause symptomless infection in plant. This method is usually done in conjunction with other confirmatory tests to achieve the maximum accuracy of viral diagnosis (Naidu *et al*., 2003).

ii. Biological Assay (Transmission Tests)

It is the one of the oldest methods for plant virus diagnosis. It is a traditional method that still uses in many laboratories as an important assay for virus detection and identification. It includes mechanical, graft, and vector transmission of the virus to susceptible herbaceous indicator plants. Mechanical transmission using sap inoculation to indicator plants can be done easily with minimum facilities. The characterizations of symptoms produced on these plants allow detection and identification of many viruses. Viruses that are not able to be transmitted via mechanical transmission as well as viruses of tree fruit can be diagnosed via vectors or grafting using suitable indicator plants. Although these assays are usually used for routine diagnosis of viruses, they may provide imprecise virus identification in addition they consume time and resources (Naidu *et al*., 2003).

iii. Microscopy

Electron Microscopy (EM) provides useful information about virus morphology. For stable viruses and viruses with filamentous and rod- shaped particles, rapid results can be observed using negative staining technique. Even though, it is not easy to detect viruses with isometric shape. Moreover, virus particles that occur in low concentration, need to be concentrated in plant sap or captured using antibody-coated grids (Immuno Sorbent Electron Microscopy) to achieve high efficiency of virus visualization. EM is commonly used for virus detection when the needed facilities are readily available, thus it cannot be used for rapid detection of multiple samples as it is very expensive and labor intensive method. Many plant viruses such as potyviruses form cylindrical inclusion bodies in infected cells. They develop large crystalline accumulations of virus particles, thus make their detection by EM a simple, rapid, and relatively inexpensive method to confirm viral infection. This cylindrical inclusions bodies are formed by a virus-encoded protein and can be considered as the most important phenotypic criterion for assigning viruses to certain genus level using selective stains (Naidu *et al*., 2003).

2.4.2 Serological Methods

Serology is one of the most easiest and specific methods for virus diagnosis that gives a rapid and precise outputs. All of serological assays depend on the virus coat protein properties and fall into two types, solid phase assays (ELISA, western immuno-blotting) and liquid phase assays (precipitation and agglutination tests). Serological techniques which include enzyme- linked immune sorbent assay (ELISA) and tissue immuno-blotting assay considered as powerful tools for plant virus detection. These techniques are based on an antigen-antibody reaction between virus specific coat proteins (epitopes) and anti-viral antibodies that raised in mammalian systems like rabbits and mice, therefore it can be visualized using several detection means such as enzyme-labelled antibodies (Hampton *et al*., 1990).

i. Enzyme-Linked Immune Sorbent Assay (ELISA)

Enzyme-linked immune sorbent assay is the most significant and popular advance among serological tests, especially in virus detection. In 1977, ELISA was introduced to plant virology by Clark and Adams. Since then it has been very common for plant virus detection in plant material, insect vectors, seeds and vegetative propagules. ELISA is widely used for the detection of plant viruses due to its simplicity, adaptability, reliability, sensitivity in addition to its economy, thus it is used to test large number of samples in a relatively short period of time (Batool *et al*., 2011 and Naidu *et al*., 2003). The basic principle of the ELISA based on immobilizing the antigen onto a solid surface, or trapping antigen by specific antibodies, and probing with specific immune globulins attached to an enzyme label. The positive reaction is detected by adding the suitable substrate. The enzyme converts substrate to product, which can be easily visualized due to its color. Many variations of ELISA have been developed. These variations are categorized into, "direct" and "indirect" forms of ELISA. Both categories have the same underlying theory and the same final results, meanwhile they differ in the way of detection for the antigen-antibody complex. The disadvantages of ELISA are the time of extraction for plant

tissue which can take several hours as well as the incubation times required for samples and antibodies in order to be adhered into microliter wells (Naidu *et al*., 2003).

ii. Tissue Blotting Immune Sorbent Assay (TBIA)

TBIA is a variation of dot-blot immuno assay (DBIA) which is used for virus detection in both plants and vectors. It was first used in 1990 (Lin *et al*., 1990). Since then it became widely used as simple and reliable method for virus detection (Chang *et al*., 2009). TBIA has relatively the same technique as ELISA except that a freshly-cut edge of plant or vector is used and spotted onto a membrane instead of using a micro titer plate. Moreover, in ELISA a soluble substrate is used for color development, while a precipitating one is usually used for virus detection in TBIA (Hsu and Lawsow *et al*., 1990). Although TBIA has some advantages over ELISA, as there is no need for tissue extraction and membranes can be blotted directly in the field and in addition to be processed at a later date, TBIA has several disadvantage is that the results are qualitative, rather than being quantitative as well as it is difficult to observe weak positive reactions due to the interference of sap components with the subsequent diagnostic reaction (Makkouk *et al*., 1994 and Naidu *et al*., 2003).

2.4.3 Nucleic Acid-Based Methods

Nucleic acid-based techniques are used extensively for plant viruses' detection and identification, mainly after the advent of the polymerase chain reaction (PCR). Although serological techniques are widely used for virus detection, they have certain drawbacks if compared with nucleic acid-based techniques. They are based on the viral coat proteins and its antigenic properties, which represents only about 10% of the total virus genome (Gould *et al*., 1983) thus they neglect the rest of the virus genome. While in nucleic acid-based detection methods any region in the virus genome can be targeted and diagnosed. Moreover, in some cases serological procedures cannot be used particularly for the detection of viroid's, satellite RNAs, viruses which lack particles (e.g.,

Groundnut rosette virus (GRV), viruses which occur as extremely diverse serotypes (e.g., Indian and African *Peanut clump virus and Tobacco rattle virus) and viruses that are difficult* to purify (Naidu *et al*., 2003). Polymerase chain reaction (PCR) is an extremely specific, sensitive and versatile in vitro method that have a great potential to amplify trace amounts of targeted nucleic acid using specific primers to the region of amplification, and thermo stable DNA polymerase (Gould *et al*., 1983 and Lepez *et al*., 2003).

It consists of three step cycles: denaturation in which the two complementary strands of the double-stranded DNA are separated at high temperature (usually 94-95°C), annealing of two oligonucleotide primers to their complementary sequences in the opposite strands of the target DNA (annealing temperature depends on the nucleotide composition and length of the primer, usually 35-65 °C), and elongation or extension of each primer through the target region (usually at 72°C) using a thermo-stable DNA polymerase (e.g., Taq polymerase). Each DNA strand made in one cycle will use as a template for synthesis of a new DNA strand in the next cycle. The procedure is repeated many times (30-40 cycles) by an automated thermal cycler until sufficient product of amplicon is produced. This procedure is directly applied for amplification of plant viruses with DNA genomes (eg. Caulimo, Gemini and Badna viruses) using gene-specific primers to the region of amplification. While in plant viruses with RNA genomes, the targeted RNA must be converted to a complimentary DNA (cDNA) using reverse transcription (RT) prior the beginning of PCR procedure. See Plate 6, In addition to its usefulness as a diagnostic technique, PCR is used by many research laboratories in the world for many purposes including, molecular characterization (Alfaro-fermade *et al*., 2008) and DNA comparisons between related pathogen species as well as in evolutionary studies (Rossink *et al.,* 2001). Although PCR can achieve a very high sensitivity and specificity, different comparative assays have been reported about a failure of PCR amplification to correctly diagnose infected and non-infected plant material. Although PCR can achieve a high

sensitivity and specificity, different comparative assays a failure of PCR amplification to correctly diagnose infected and non- infected plant material has been reported. This failure could be a consequence of the "carry-over" contamination of amplicons that can be responsible for false-positive results and inhibitor components in sample extracts which is the main reason for false negatives (Naidu *et al*., 2003). Nucleic acid detection and serology apprehension techniques usually acclimatize to analyze and characterize suspected viral diseases. Molecular tests, like RT-PCR and serological tests (Cocktail and Sandwich ELISA) are used to diagnose *PLRV*. Khouadja *et al.*, (2003) studied to detect *PLRV* using RT-PCR and sandwich ELISA in 131 potatoes sampled from different location of Tunisia, and proved that RT-PCR is more reliable and sensitive with added advantage of less time involved than serological tests. For routine laboratory diagnosis to detect the *PLRV* various molecular methods (like RT-PCR) can be used because is reliable, robust, and highly sensitive in a short time (Schoen *et al.*, 1996).

CHAPTER III

MATERIALS AND METHODS

MATERIALS & METHODS

Disease plays an important role in reducing the quality and quantity of cultivated crops. The present study was conducted to study incidence and severity of potato viruses in three selected districts of Bangladesh and their molecular detection through RT-PCR based analysis. This chapter deals with three experiments throughout the study period in order to study the major potato viral diseases. The experiments were as follows-

Experiment-I: Collection of diseased samples and determination of disease incidence (DI) and disease severity (DS) of major potato viruses from three selected districts of the northern region of Bangladesh

Experiment-II: Detection of the major potato virus through RT-PCR

3.1 Experiment-I: Collection of Disease Samples and Determination of Disease Incidence (DI) and Disease Severity (DS) of Major Potato Viruses from Three Selected Districts of The Northern Region of Bangladesh

3.1.1 Location of Surveyed Area

Incidence and severity of virus diseases occurred on potato leaves raised in the selected areas was surveyed. Incidence of major potato viruses in three major potato growing areas of the northern region of Bangladesh, viz. Bogura, Gibandha and Jaipurhat were investigated in field samples. Overall, three upazila (sub-station) from each district (which were at about 20 km apart) and three spots under each upazila were selected for samples collection, disease incidence and disease severity monitoring. Ten samples (5 random and 5 non-random) were collected from each upazila. A total of 270 samples were collected on the basis of virus and viral like symptoms.

3.1.2 Survey Period

Surveys were made during the period of December, 2019. Survey was done in three selected upazila from each of the selected district.

3.1.3 Data Collection

Data were collected from farmer's field during potato cultivation to observe disease incidence and disease severity. Data were collected from the selected locations followed by a prepared simple questionnaire. Data was collected by the following process.

i) Non Random Sampling

Definition: Non random sampling is a sampling technique where the samples are gathered in a process where all the individuals in the population does not give equal chances of being selected (Figure 3).

Figure 3. Non-random sampling process from potato field to collect virus infected sample for further analysis

ii) Random sampling

Definition: Random sampling is a part of the sampling technique in which each sample has an equal chance of being chosen. A sample chosen randomly is meant to be an unbiased representation of the total population (Figure 4).

Figure 4. Random data sampling from potato field to collect virus infected

3.1.4 Observation of the Symptoms

Symptoms of the potato diseases were studied by visual observation. Sometimes a disease was identified based on matching the observed symptoms in the infected leaves with the symptoms. Identification of all the virus diseases was finally confirmed by identification of the associated virus organism through isolation.

3.1.5 Collection of Diseased Specimen and Processing

Potato leaves were collected from selected upazila through random and non-random sampling procedure. The specimens were collected in zip lock back and kept in ice box and preserved for few hours and then it kept in the laboratory room following standard procedure of preservation. The disease specimen was used for the RNA extraction. The collected disease specimens ware arranged in district wise that represent the Plate 3.

a) *PLRV* infected sample collection

b) *PVX* infected sample collection

c) *PVY* infected sample collection

d) Samples from Bogura districts

e) Samples from Jaipurhat districts

f) Samples from Gaibandha districts

Plate 3. Collection of diseased specimen and processing. a) *PLRV* infected sample collection b) *PVX* infected sample collection c) *PVY* infected sample collection d) Samples from Bogura districts e) Samples from Jaipurhat districts f) Samples from Gaibandha districts

3.1.6 Data Collection during Survey

During the survey in the selected study areas, total numbers of infected plant were counted. Primary identification disease was done by visual observation.

3.1.7 Determination of Disease Incidence

Data were collected from farmer's field during potato cultivation to observe disease incidence and severity of potato viruses. Randomly and non- randomly 500 m^2 (25m × 20m) area were selected in farmer field. Total leaves were counted from 500 m^2 selected area and among them disease infested leaves were counted to calculate percent disease incidence. The disease incidence was calculated by using the following formula.

$$\textbf{Disease incidence (\%)} = \frac{\text{Number of leaf samples positive for a virus}}{\text{Total number of leaves tested}} \times 100$$

3.1.8 Calculation of Disease Severity

The plants were scored for disease class on a scale of 0 to 4 (Table 2). The Disease Severity (DS) was calculated using a modified method of Islam, 2015. The DS was calculated based on the following formula:

$$\textbf{Plant Disease Index (PDI)} = \frac{\text{Some of all disease rating}}{\text{Total no of disease rating} \times \text{maximum no of rating}} \times 100$$

Table 2. Disease rating scale for *PLRV, PVY, PVX* (Islam, 2015)

Index	*PLRV*	*PVY*	*PVX*	Reaction
0	No visible symptoms	No symptoms	No visible symptoms	Highly resistance
1	Rolling of leaves in case of primary infection and lower leaves in case of secondary infection, erect growth	Blackening and banding of vein on few leaves. Mosaic starting on all leaves.	Mild mottling on the upper leaves	Resistance
2	Rolling of leaves extending, leaves become stiff and leathery, stunting of plants and erect growth	Blackening and banding of vein on all leaves. Narrowing of leaves. Veinal necrosis, severe mosaic, leaf crinkling	Inter veinal mosaic symptoms on more than one leaves.	Moderately resistance
3	Short internodes, papery sound of leathery leaves, rolling and stunting of whole plants. Young buds are slightly yellowish and purplish	Rugosity and leaf drop streak, dwarfing	Mosaic symptoms on all leaves.	Moderately susceptible
4	Clear rolling of leaves, severe stunting, few tubers and tuber necrosis	Lower leaves dead, drooping collapse of plants with very small tubers	Distinct mosaic symptoms on all leaves	Susceptible

3.1.9 Data Analysis

The collected data was arranged then put in the excel sheet and analysis by using the computer basis software Statistix 10 at 0.05 level of significance.

3.2 Experiment-II: Detection of the Major Potato Virus through RT-PCR

After the survey of selected districts, *PLRV* was found to be the most predominant virus. So, we had decided to select *PLRV* for molecular detection.

3.2.1 Experimental Site

The experiment was conducted in Molecular Biology and Plant Virology Laboratory under the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207.

3.2.2 Collection of *PLRV* +ve Samples and Maintenance

PLRV +ve samples were collected from infected potato plants from selected upazila on the basis of *PLRV* typical symptoms which include mainly rolled leaves.

3.2.3 Multiple Sequence Alignments for Primer Designing

For designing the accurate and specific primers for amplification through RT-PCR, we aligned the subject *PLRV-CP* gene sequence with the reported reference sequenced from National Centre of Biotechnology Information (NCBI). We selected the *PLRV-CP* gene sequence (*Accession number NC_001747.1, accessed on 2018/12/31*) and aligned with 56 reported sequences. For further analysis and alignment, we took sequences with maximum homology (>96%) and query coverage (>97%) to our query sequence. We used Basic Local Alignment Search Tool (BLAST) for searching out the homologous sequence at Gene Bank nucleotide database. According to our criteria, we selected total 16 sequences. These sequences were aligned with ClustalW2. The procedure proceeded with pairwise alignment followed by multiple alignments. The gap penalty and other parameters, for calculating the best alignment, were kept default. The deduced, computationally, protein sequence was also aligned in the same method, first pairwise and then multiple alignments.

3.2.4 Primer Designing

Specific to CP gene of *PLRV* primers were designed with the help of a software primer-3 version 0.4.0 (Steve and Skaletsky, 2000). Gene Bank "http://frodo.wi.mit.edu/primer3/", verified on 2018/04/15, *"Accession number NC b001747*". Multiple sequence alignments were done by using Clustal-W (Larkin *et al.,* 2007) to identify the conserved regions. Primers were made to amplify the conserved/less mutating genomic segment and tested for primer specificity *in-silico* by applying BLAST, provided by, to reduce the chance of non-specificity. The 3′ sequence primers with no similarity to viral sequences or other origin sequences were marked as selected. Primers were synthesized commercially and presented in Table 3.

Table 3. Primer sequence used to amplify 346bp fragment of PLRV-Coat protein (CP) gene

Primers	Primer Sequence (5'-3')	Amplicon position (nt)	Amplicon size (bp)	TºC	GC Contents (%)
PLRV 346-FP	CAGGCGCCGAAGACGCAGAA	3693-4319	346	60.04	65.00
PLRV 346-RP	TTTGGCGCCGCCCTTCGTAA			59.63	60.00

3.2.5 RNA Extraction from *PLRV* +ve Leaf Samples

PLRV is an ssRNA virus. Total RNA extracted from the collected *PLRV*+ve plant leaves by the "single-step" method as described by following flow chart (Figure 5, Chomczynski and Sacchi, 1987).

Collected leaves were dipped into liquid N_2 and ground to fine powder with already cooled pestle and mortar

⇩

Ground samples were shifted to 1.5 ml tubes

⇩

Trizol @ one ml per 0.2g of ground samples was added and kept for 5 minutes at room temperature for dissociation of nucleoprotein complex, completely.

⇩

Chloroform 0.2 ml per 1 ml of Trizol was poured before through shaking for 30-45 seconds.

⇩

Then, it was put at room temperature for 5-10 minutes.

⇩

After this incubation, centrifugation on 13000 rpm for at 4°C 15 minutes was given.

⇩

The RNA rich upper portion of the centrifuged solution was poured into another eppendorff.

⇩

The precipitation of RNA was achieved by pouring 0.5 ml isopropanol.

⇩

Ten minutes of incubation time was given to samples followed by centrifugation for 10-12 minutes with 13000 rpm at 4°C.

⇩

Washing of pellet was done by adding 1ml ethanol (75%) and supernatant was removed.

⇩

The sample was re-suspended properly through pipetting and centrifuged at 10000 speeds for 5-6 minutes and temperature was 4°C.

⇩

Then remove supernatant and air dry for 10 minutes time of RNA pellet.

⇩

DEPC treated water (20µl) was used to resuspend RNA and put at -20°C. After quantification through spectrophotometer RNA quality was confirmed at 1% agarose gel

Figure 5: Flow chart of RNA extraction from *PLRV*+ve leaf samples

a) Sample grinding

b) Grounded samples with 0.2g Trizol

c) Adding Chloroform

d) Centrifugation

e) Adding Isopropanol

f) Washing pellet with Ethanol

g) Drying pellets

h) Re-suspending RNA with DEPC treated water

Plate 4. Steps of RNA Extraction. a) Sample grinding b) Grounded samples with 0.2g Trizol c) Adding Chloroform d) Centrifugation e) Adding Isopropanol f) Washing pellet with ethanol g) Drying pellets h) Re-suspending RNA with DEPC treated water

3.2.6 Confirmation of the Extracted RNA

Total RNA was extracted from *PLRV*+ve leaves of potato following the "single-step" method as described in methodology section. RNA is sensitive to temperature. High temperature denatures RNA property. That is why, the extracted RNA was subjected to use for cDNA synthesis that was stored in -20^{O}C refrigerator for further use. Before cDNA synthesis, RNA was quantified in 1% agarose gel and observed under UV lights. Results are presented in Figure 6.

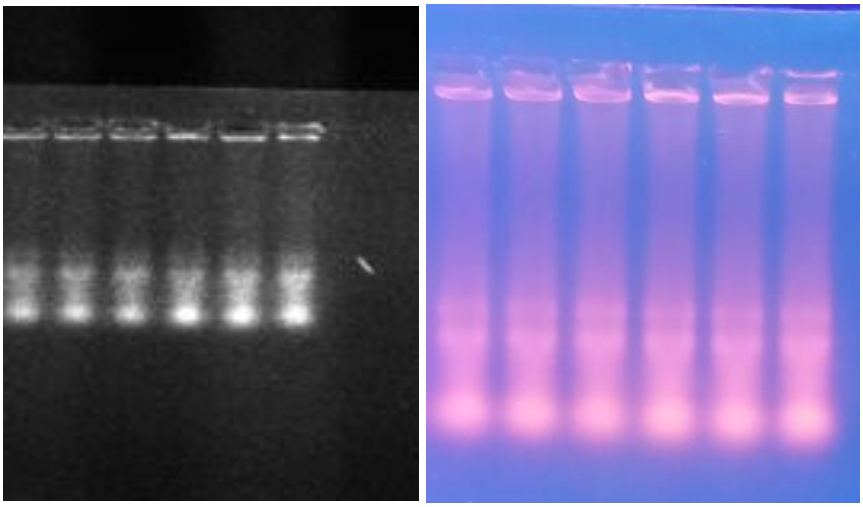


Figure 6. RNA from infected leaves of potato analyzed at 1% agarose gel.

3.2.7 Complementary DNA (cDNA) Synthesis

"First Strand cDNA Synthesis kit" (Biolabs) was used to synthesize cDNA. The cDNA was synthesized by adding the total RNA @ 1µg plus 2.0µl of the random primer mix i.e.20 pmoles plus nuclease-free water to make final volume at 8µl and mixed slightly and spun gently in a micro-centrifuge for 3-5 seconds. After, giving 5 minutes of incubation at 65^{o}C plus ice chilling plus addition, 2X reaction mix at 10µl plus 10X Enzyme mix at 2µl (20u/µl) then incubation at 25^{o}C, 5 minutes. Lastly, incubation was done at 42^{o}C plus 60 minutes and 80^{O}C for 5 minutes and stored at -20^{O}C. The thermo cycling condition was as follows (Figure 7).

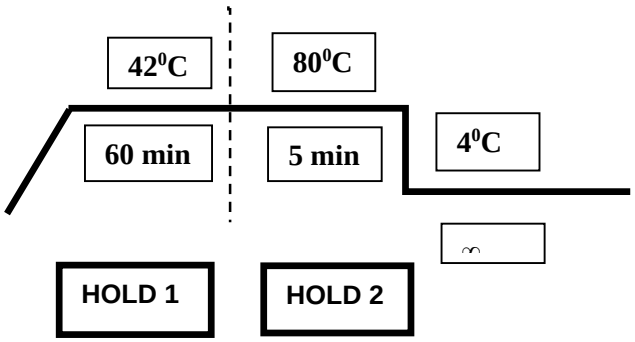


Figure 7. Cycling profile for complementary DNA (cDNA) synthesis

a) cDNA synthesis kit **b) PCR machine**

Plate 5. cDNA synthesis equipment. a) cDNA synthesis kit b) PCR machine

3.2.8. RT–PCR Amplification

The 346 bp product was visualized from cDNA of *PLRV* by using pair of primers. The reaction mixture of PCR is attaches as Appendix-II. PCR conditions are elaborated as shown in the Figure 8.

Forward primer (FP): 5'-CAGGCGCCGAAGACGCAGAAA-3'

Reverse primer (RP): 5'-TTTGGCGCCGCCCTTCGTAA-3'

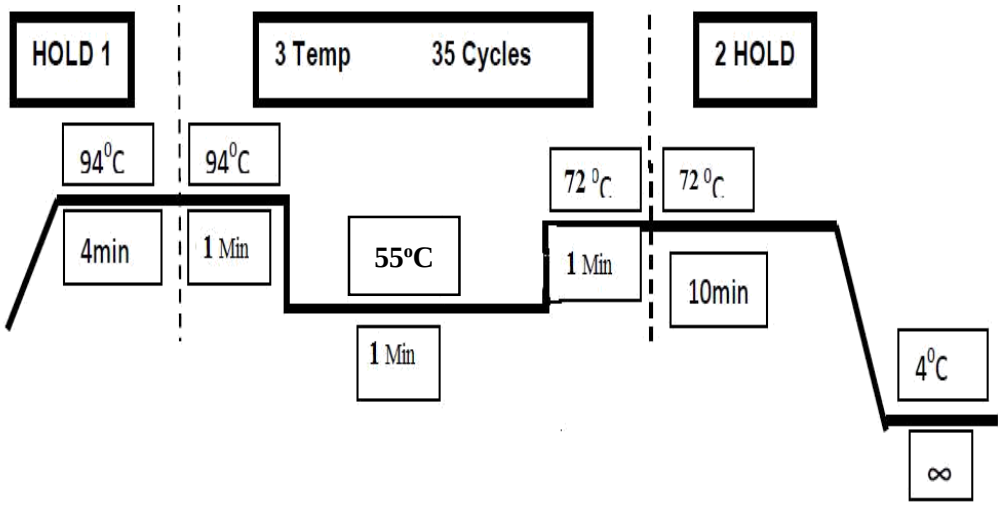


Figure 8. RT-PCR Cycling Conditions to Amplify *PLRV-CP* Gene Fragment

a) PCR mixture preparation **b) PCR amplification**

Plate 6. Process of PCR amplification. a) PCR mixture preparation b) PCR amplification

Table 4. The composition of 20 µl PCR reaction

Components	Volume
10x PCR buffer	2 µl
1m M dNTPs	2 µl
FP (10pmoles)	2 µl
RP (10pmoles)	2 µl
Template (cDNA)	4 µl
Taq polymerase	0.5 µl
PCR H_2O	Up to 20 µl

3.3.9. Agarose Gel Electrophoresis

Total 1% of Agarose gel mixed with TBE buffer 1X helped to resolve the RT-PCR products. To confirm the product size of the RT-PCR, 100 bp DNA ladder (Thermas) was used as marker. Before visualizing 346 bp product under UV light, gel was stained with ethidium bromide.

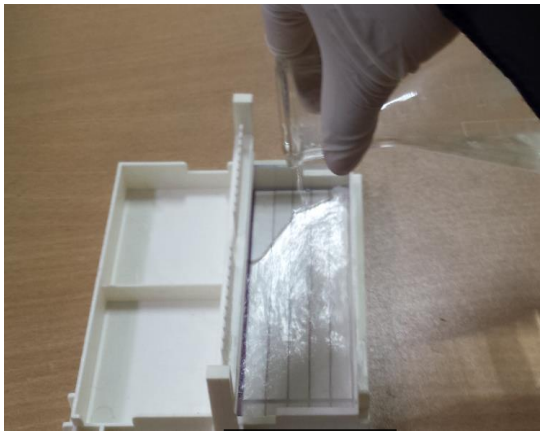
Table 5. Composition of 500ml 5X TBE Buffer

Tris-HCl	27 gm
Boric acid	13.75 gm
EDTA	2.325 gm
NaOH pellets	As required (upto pH 8)
Distilled water	Up to 500ml

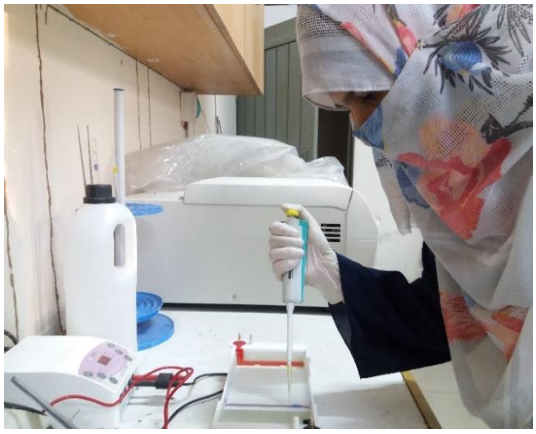
For preparing 500ml 1X TBE buffer, 400 ml distilled water was added to100 ml 5X TBE buffer.

a) TBE buffer ingredient

b) TBE buffer preparation



c) 1% Gel preparation



d) loading in 1% agarose gel

Plate 7. Steps in agarose gel electrophoresis. a) TBE buffer ingredient b) TBE buffer preparation c) 1% Gel preparation d) loading in 1% agarose gel

CHAPTER IV

RESULTS AND DISCUSSION

RESULTS

Disease is one of the major constrains to successful crop production. Whereas viral disease can cause the most severe yield loss. Viruses generally occurs in phloem region of plants. Potato viruses mainly occur in leaves that causes rolling, curling, chlorosis, mottling, necrosis, stunting etc. of potato leaves. The deformed leaves hamper in photosynthesis and that finally cause hinder to the formation of quality tuber production. This chapter includes the result of the study i.e. disease incidence and severity of major potato viruses in selected locations, molecular detection of the predominant potato virus of the survey i.e. *PLRV* through Reverse Transcriptase Polymeric Chain Reaction (RT-PCR). Symptomatology of the major potato virus, vector association of the plant leaves of potato viruses was also studied.

4.1. Symptoms of the Major Viral Diseases of Potato and Identification on The Basis of Visible Characteristics Symptoms

4.1.1. Symptomatology

Stunting, systemic vein clearing, mosaic, mottling, curling, shortening of leaves, dark green and vein-banding were observed one of the potato plants. In some plants with leaf roll symptoms, entire leaflets were rolled with chlorosis (yellowing), reddening, 'leathering' of leaves and stunting infected plants which are characteristics symptoms of potato leaf roll disease that caused by *PLRV* (Plate 8). In some plants with mosaic symptoms of young leaves, mottling, shortening of leaves, dark green and stunting infected plants, these are the prominent symptoms of severe mosaic of potato caused by *PVY* (Plate 9). In few plants with systemic vein clearing and vein-banding was observed this is the characteristics symptoms of mild mosaic that caused by *PVX* (Plate 10).

a) leaf rolling (initial)

b) leaf leathering (severe)

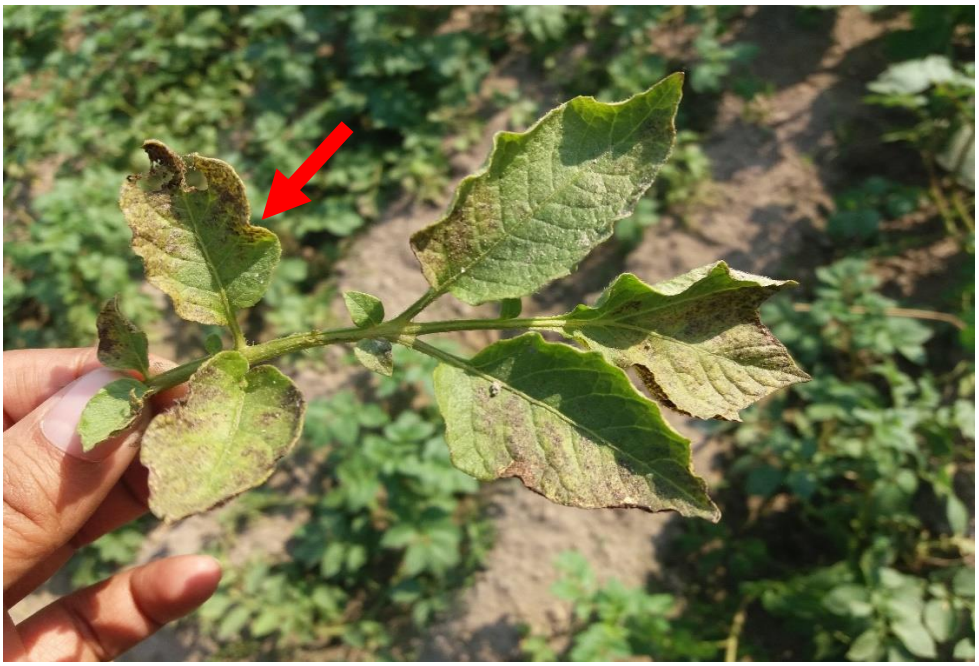
Plate 8. Foliar symptoms of *PLRV* include a) leaf rolling (initial) b) leaf leathering (severe)

a) severe mosaic of young shoots (initial)

b) stunting infected plant (severe)

Plate 9. Foliar symptoms of *PVY* include with a) severe mosaic of young shoots (initial) b) stunting infected plant (severe)

a) mottling of leaves (initial)



b) leaf necrosis (severe)

Plate 10. Foliar symptoms of *PVX* include a) mottling of leaves (initial) b) leaf necrosis (severe)

4.1.2. Insect Vectors Association with Potato Leaves in Virus Infected Plants

Vector association in infected fields was also studied. After the survey, it was determined that *PLRV* and *PVY* were transmitted by the insect vector aphid (Figure 9 & 10). But *PVX* which is mechanical transmitted is not transmitted by aphid. Aphid generally lies beneath the potato leaves.

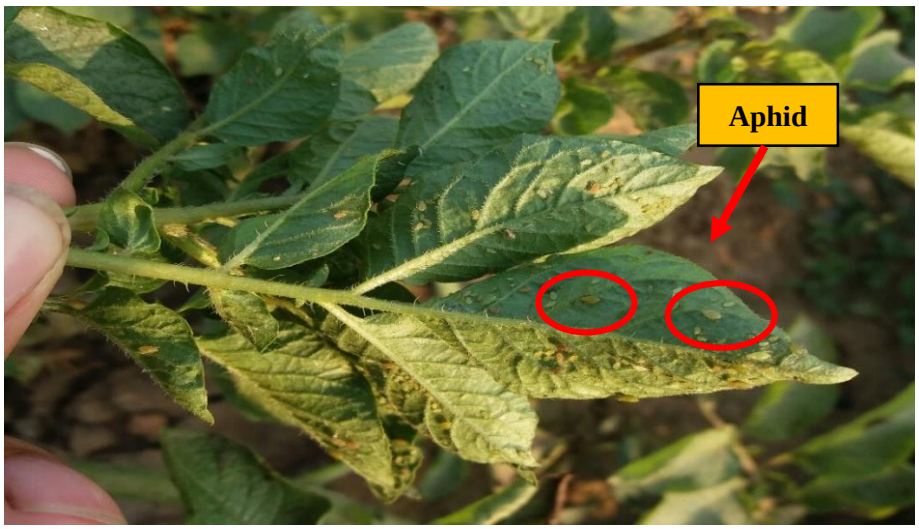


Figure 9. Association of insect vector aphids in *PLRV* infected plant

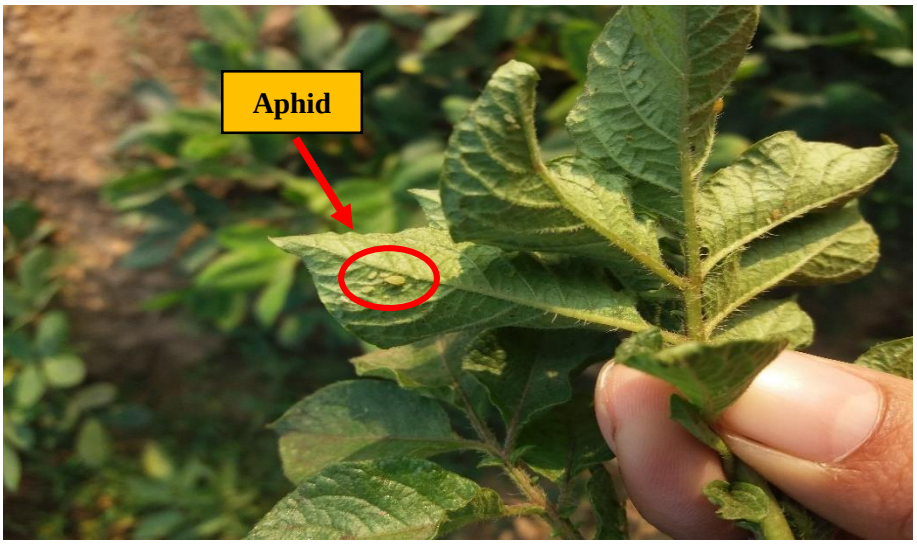


Figure 10. Association of insect vector aphids in *PVY* infected plant

4.2. Survey Study

The survey was conducted in three selected districts in Bangladesh viz. Bogura, Jaipurhat and Gaibandha. Three upazilas were selected from each of the selected districts. Data were collected in both random and non-random sampling procedures. In total 270 samples were considered for calculating disease incidence (%DI) and disease severity (%DS).

4.2.1. Potato Varieties Cultivated in Selected Upazila in Bangladesh Tuber Used and Yield in Selected Upazila of Bogura Districts

In Bangladesh, both hybrid and local varieties of potato are cultivated. Most of the farmers are interested to cultivate hybrid cultivars due to get higher production. From our survey study, it was noticed that most of the farmers selected hybrid varieties for commercial cultivation. Although the local varieties have the popularity of taste. In Shibgong upazila of Bogura district, total cultivated area was 18,500 ha of land and production was 4, 14,955 mton (22.43 MT/Ha). Out of this cultivated area mostly occupied by hybrid varieties. For example, Asterics (5,500 ha), Cardinal (2,500), Granular (2,500). Among the local varieties, Pakri (4,200 ha) was mostly cultivated in this upazila. In Shajahanpur upazila of Bogura district, total cultivated area was 5,270 ha of land and production was 1, 16,935 mton (22.19 MT/Ha). Out of this cultivated area mostly occupied by hybrid varieties. For example, Diamond (1,400 ha), Granular (1,500), Asterics (800 ha). Among the local varieties, Pakri (700 ha) was mostly cultivated in this upazila. In Sadar upazila of Bogura district, total cultivated area was 6,800 ha of land and production was 1, 47,337 mton (21.67 MT/Ha). Out of this cultivated area mostly occupied by hybrid varieties. For example Diamond (1,500 ha), Granular (1,700 ha), Asterics (700 ha). Among the local varieties, Pakri (840 ha) was mostly cultivated in this upazila. From the study regarding the potato varieties cultivated in Bogura district, it was observed that hybrid varieties are mostly cultivated in this district but most of the hybrid varieties are highly susceptible to potato viruses. The survey data are presented in Table 6.

Table 6. Potato varieties cultivation per area in main potato growing upazila in Bogura district (2019-2020).

Sl No	Upazila	Potato variety	Area (Ha)	Yield (MT)	Yield Rate (MT/Ha)
1	**Shibgonj**	Asterics	5500	**4,14,955**	**22.43**
		Cardinal	2500		
		Granular	2500		
		Diamond	1000		
		Carriage	500		
		Romana	360		
		Pakri	4200		
		Hagrai	200		
		Musica	300		
		Destiny	200		
		Alvery	400		
		Clear	100		
		Roseta	100		
		Others	440		
		Total	**18,500**		
2	**Shajahanpur**	Diamond	1400	**1,16,935**	**22.19**
		Cardinal	300		
		Granular	1500		
		Asterics	800		
		Carriage	300		
		Hagrai	90		
		Pakri	700		
		Others	180		
		Total	**5,270**		
3	**Sadar**	Diamond	1500	**1,47,337**	**21.67**
		Cardinal	500		
		Granular	1700		
		Asterics	700		
		Carriage	400		
		Musica	200		
		Hagrai	550		
		Pakri	840		
		Others	390		
		Total	**6,800**		

Source: DAE, Bogura.

4.2.2. Potato Varieties Cultivated in Selected Upazila in Bangladesh Tuber Used and Yield in Selected Upazila of Jaipurhat Districts

In Kalai upazila of Jaipurhat district, total cultivated area was 10,800 ha of land and production was 2, 39,868 mton (22.21 MT/Ha). Out of this cultivated area mostly occupied by hybrid varieties. For example, Asterics (4,940 ha), New zika (2,500), Romana (1,280). In Khetlal upazila of Jaipurhat district, total cultivated area was 8,400 ha of land and production was 1, 84,632 mton (21.98 MT/Ha). Out of this cultivated area mostly occupied by hybrid varieties. For example, Diamond (2,110 ha), Granular (1,490), Asterics (3,000 ha). Among the local varieties, Pakri (400 ha) was mostly cultivated in this upazila. In Sadar upazila of Jaipurhat district, total cultivated area was 6,800 ha of land and production was 1, 48,104 mton (21.78 MT/Ha). Out of this cultivated area mostly occupied by hybrid varieties. For example, Diamond (1,650 ha), Granular (1,100 ha), Asterics (2.200 ha). Among the local varieties, Pakri (360 ha) was mostly cultivated in this upazila. From the study regarding the potato varieties cultivated in Jaipurhat district, it was observed that hybrid varieties are mostly cultivated in this district but most of the hybrid varieties are highly susceptible to potato viruses. The survey data are presented in Table 7.

Table 7. Potato varieties cultivation per area in main potato growing upazila in Jaipurhat district (2019-2020)

Sl No	Upazila	Potato variety	Area (Ha)	Yield (MT)	Yield rate (MT/Ha)
1	**Kalai**	Asterics	4,940	**2,39,868**	**22.21**
		New Zika	2,500		
		Granular	780		
		Carriage	480		
		Romana	1,280		
		Musica	400		
		Cardinal	200		
		Others	220		
		Total	**10,800**		
2	**Khetlal**	Diamond	2,110	**1,84,632**	**21.98**
		Cardinal	450		
		Granular	1,490		
		Asterics	3,000		
		Carriage	600		
		Romana	85		
		Pakri	400		
		Musica	150		
		Others	115		
		Total	**8,400**		
3	**Sadar**	Diamond	1,650	**1,48,104**	**21.78**
		Cardinal	400		
		Granular	1,100		
		Aserics	2,200		
		Carriage	540		
		Romana	85		
		Pakri	360		
		Musica	110		
		New Zika	100		
		Others	255		
		Total	**6,800**		

Source: DAE, Jaipurhat.

4.2.3. Potato Varieties Cultivated in Selected Upazila in Bangladesh Tuber Used and Yield in Selected Upazila of Gaibandha Districts

In Gobindogonj upazila of Gaibandha district, total cultivated area was 5,050 ha of land and production was 97,550 mton (19.32 MT/Ha). Out of this cultivated area mostly occupied by hybrid varieties. For example, Asterics (2,270 ha), Romana (460), Granular (400). Among the local varieties, Pakri (200 ha) was mostly cultivated in this upazila. In Polashbari upazila Gaibandha district, total cultivated area was 1,120 ha of land and production was 22,814 mton (20.37 MT/Ha). Out of this cultivated area mostly occupied by hybrid varieties. For example, Asterics (350 ha), Cardinal (150 ha) and Carriage (150). Among the local varieties, Pakri (85 ha) was mostly cultivated in this upazila. In Sadar upazila, total cultivated area was 320 ha of land and production was 6,400 mton (20 MT/Ha). Out of this cultivated area mostly occupied by hybrid varieties. For example, Asterics (120 ha), Cardinal (45 ha) and Musica (40 ha). Among the local varieties, Pakri (35 ha) was mostly cultivated in this upazila. From the study regarding the potato varieties cultivated in Gobindogonj district, it was observed that hybrid varieties are mostly cultivated in this district but most of the hybrid varieties are highly susceptible to potato viruses. The survey data are presented in Table 8.

Table 8. Potato varieties cultivation per area in main potato growing upazila in Gaibandha district (2019-2020)

Sl No	Upazila	Potato variety	Area (Ha)	Yield (MT)	Yield rate (MT/Ha)
1	**Gobindogong**	Cardinal	250	**97,550**	**19.32**
		Asterics	2,270		
		Granular	400		
		Carriage	250		
		Musica	350		
		Romana	460		
		New Zika	150		
		Hira	100		
		Pakri	200		
		Others	220		
		Total	**5050**		
2	**Polashbari**	Cardinal	150	**22,814**	**20.37**
		Asterics	350		
		Granular	70		
		Carriage	150		
		Musica	85		
		Romana	45		
		Hira	55		
		Pakri	85		
		Others	130		
		Total	**1120**		
3	**Sadar**	Cardinal	45	**6,400**	**20**
		Asterics	120		
		Granular	30		
		Carriage	25		
		Musica	40		
		Romana	15		
		Pakri	35		
		Others	10		
		Total	**320**		

Source: DAE, Gaibandha.

4.2.4. Sources of Seed Tubers, Seed Treating Fungicides and Pesticide Used in the Surveyed Areas

In the survey study, we also collected the data on sources of seed tubers, seed treating fungicides and pesticides. Pesticides are mainly used to control the insect vectors. It was observed that sources of seed tuber were mainly cold storage, govt. organization (BADC) and home storage. Some farmers also collect the seed from company sources. It was also observed that for seed tuber treatments most of the farmer use Provax, Carbendazim, Thiram and Mancozeb. Farmers were also use ash for seed tuber treatment as a conventional practice. Use of insecticides was also observed for quality potato production in selected districts. Insecticides are being used to control the insect vector for plant viruses. Most of the farmers used Cypermethrin, Emidachloromite, Chloropyriphos. The survey information are presented in Table 9.

Table 9. Source of seed tuber, Seed tuber treating agents, Insecticide used in the selected districts

Sl No	Districts	Source of seed tuber	Seed tuber treating agents	Insecticide used
1	Bogura	Cold storage	Provax	Emidachloromite
		Govt. Organization	Carbendazim	Cypermethrin
		Home storage	Ash	
2	Jaipurhat	Govt. organization	Provax	Emidachloromite
		Cold storage	Carbendazim	Cypermethrin
		Home storage	Mancozeb	Chloropyriphos
		Company seeds	Thiram	
3	Gaibandha	Cold storage	Provax	Cypermethrin
		Home Storage	Ash	Chloropyriphos
		Govt organization		

Source: DAE of Bogura, Jaipurhat and Gaibandha.

4.2.5 Disease Incidence and Severity of Selected Districts

Disease is an unwanted event in any crop production. It is one of the major threats in desired crop yield. Disease incidence and severity are the main parameter to calculate crop loss assessment. On the basis of visual observation, in total 131 samples were found to be infected with virus and virus like symptoms out of 270 samples. *PLRV*, *PVY* and *PVX* infected samples were counted 72, 52 and 7 respectively. DI and DS of the major potato virus in selected upazilas were calculated, compared and presented in graphical form. During data collection the temperature and humidity were also observed.

4.2.5.1 The Disease Incidence and Severity of *PLRV, PVX* and *PVY* in Surveyed Districts

From the study in Bogura districts, it was observed that among the considerable no of samples the highest disease incidence (30.67%) and severity (25.67%) was calculated in case of *PLRV*. In case of *PVY* the disease incidence (20.33%) and severity (16.67%) was moderate. The lowest disease incidence (2.5%) and severity (2.5%) was calculated in case of *PVX*. During the survey, the average temperature and humidity was 17^{o}C and 65% respectively as clear in Figure 10. In case of Jaipurhat districts, it was observed that among the considerable no of samples the highest disease incidence (23%) and severity (17%) was calculated in case of *PLRV*. In case of *PVY* the disease incidence (15%) and severity (8.9%) was moderate. The lowest disease incidence (2.33%) and severity (1.43%) was calculated in case of *PVX*. During the survey, the average temperature and humidity was 18^{o}C and 68% respectively as clear in Figure 11. In Gaibandha districts, it was observed that among the considerable no of samples the highest disease incidence (23.3%) and severity (18.67%) was calculated in case of *PLRV*. In case of *PVY* the disease incidence (18%) and severity (13%) was moderate. The lowest disease incidence (2.3%) and severity (1.33%) was calculated in case of *PVX*. During the survey, the average temperature and humidity was 16^{o}C and 60% respectively as clear in Figure 12.

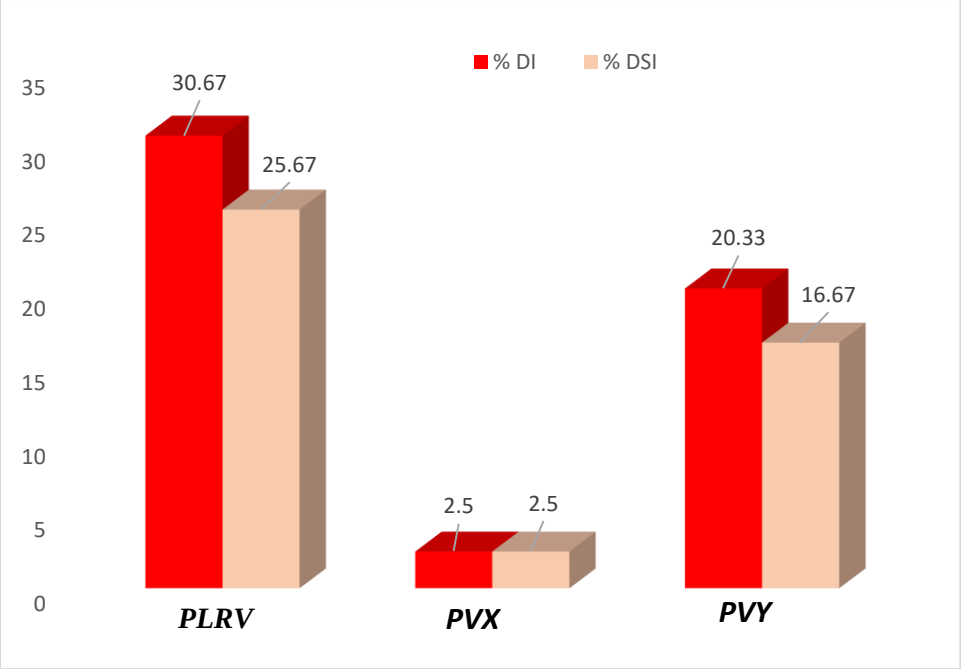


Figure 11. Disease incidence and disease severity of potato viruses in Bogura

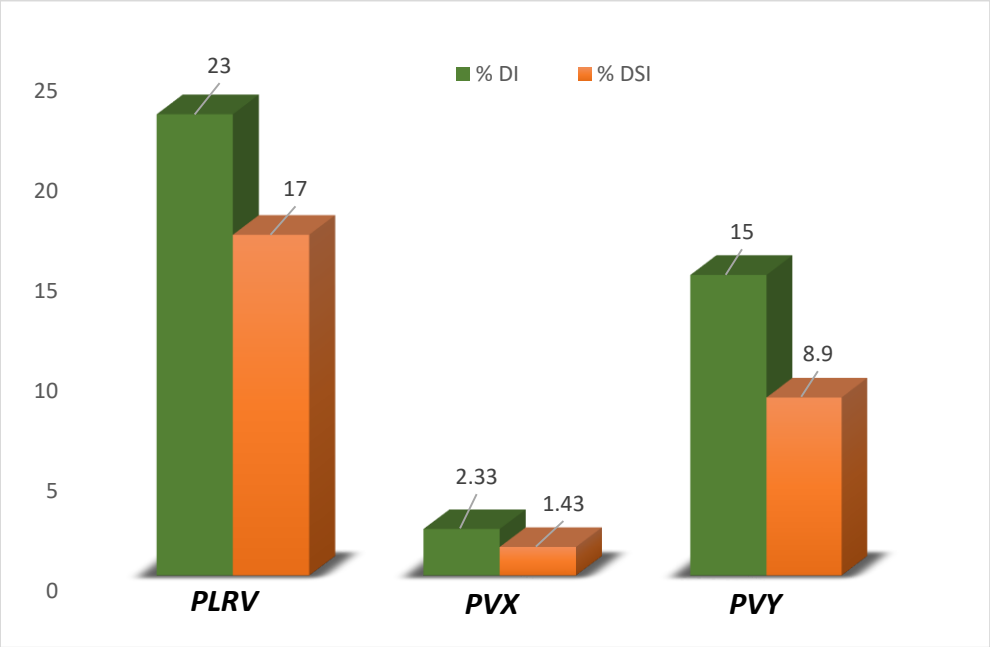


Figure 12. Disease incidence and disease severity of potato viruses in Jaipurhat

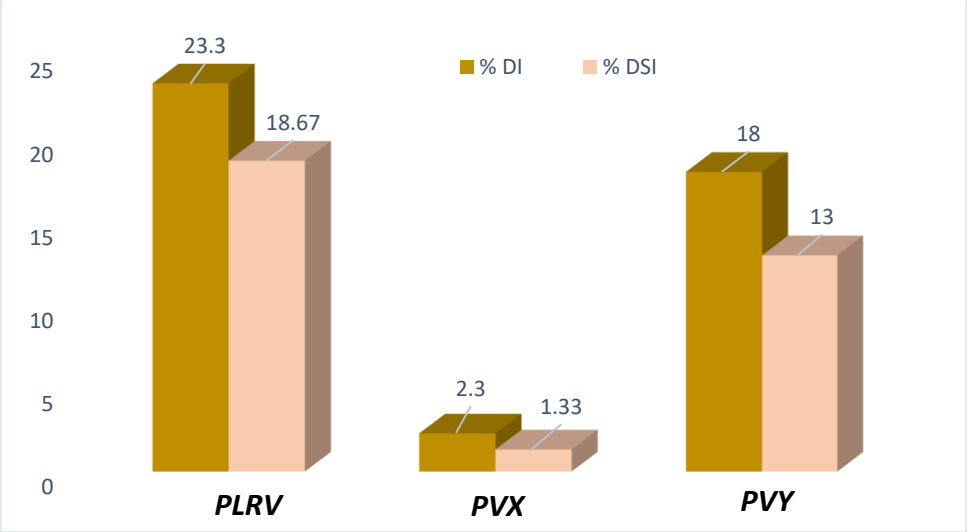


Figure 13. Disease incidence and disease severity of potato viruses in Gaibandha

4.2.5.2 District Wise Comparison of Disease Incidence and Severity for *PLRV, PVX* and *PVY*

In case of *PLRV* the disease incidence and severity was higher in all selected districts. The highest disease incidence (27.67%) and severity (25.67%) of *PLRV* was found in Bogura districts and the lowest disease incidence (23%) and severity (17%) was found in Jaipurhat district which is statistically similar with Gaibandha district viz. 23.33% and 18.67% respectively as clear in the Figure 13. In case of *PVY*, the disease incidence and severity was moderate in all selected districts. The highest disease incidence (20.33%) and severity (17.67%) of *PVY* was found in Bogura districts and the lowest disease incidence (15.33%) and severity (8.9%) was found in Jaipurhat district which is statistically similar with Gaibandha district viz. 18% and 13% respectively as clear in the Figure 14. In case of *PVX*, the disease incidence and severity was lower in all selected districts. The highest disease incidence (3%) and severity (2.33%) of *PVX* was found in Bogura districts and the lowest disease incidence (2%) and severity (1.43%) was found in Jaipurhat district which is statistically similar with Gaibandha district viz. 2.33% and 1.33% respectively as clear in the Figure 15 & 16.

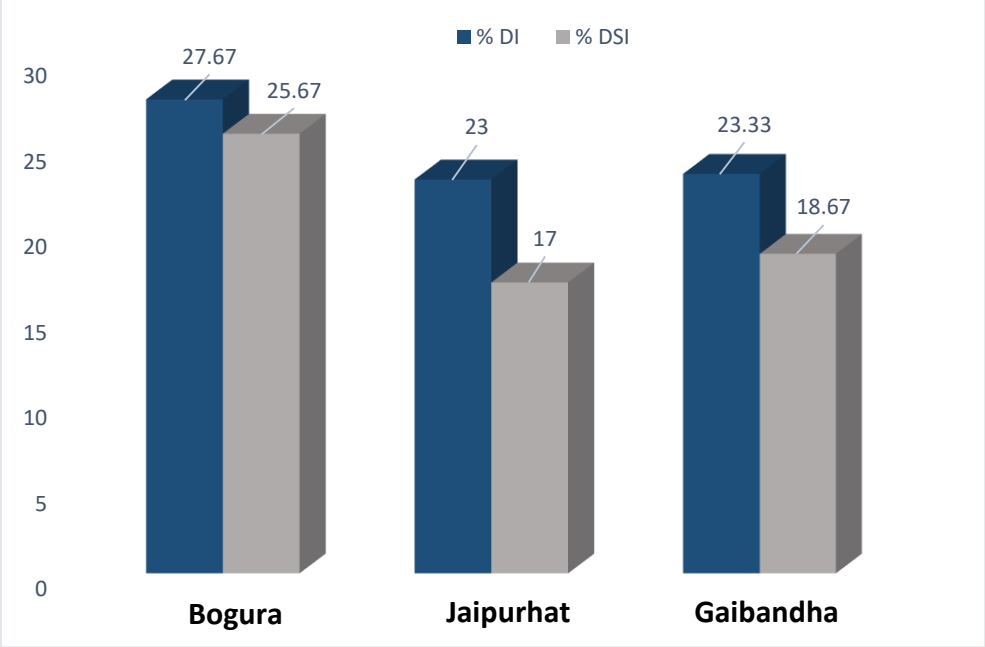


Figure 14. Disease incidence and disease severity of *PLRV* in selected districts

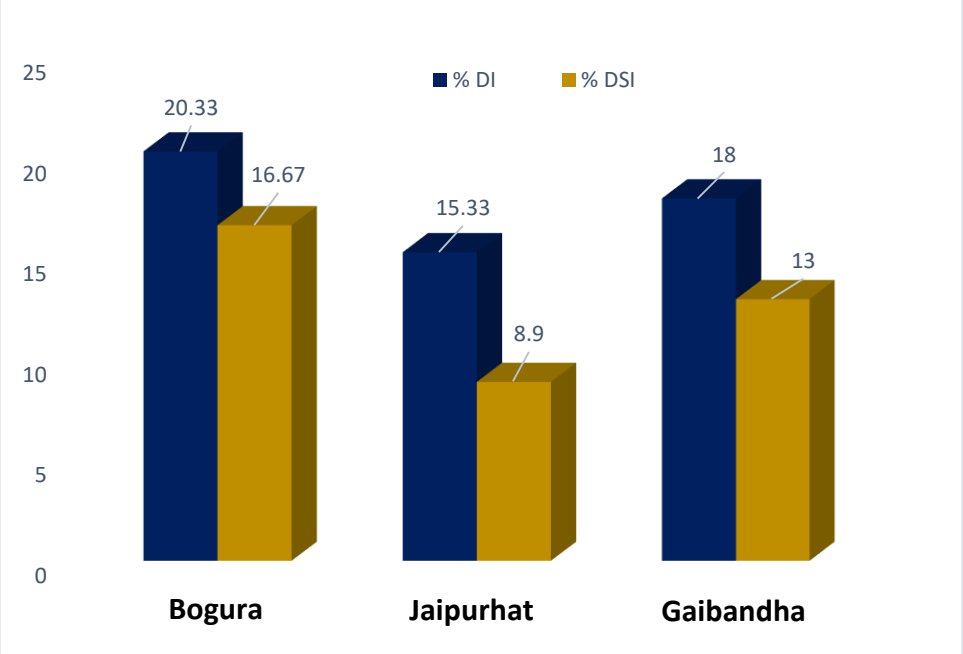


Figure 15. Disease incidence and disease severity of *PVY* in selected districts

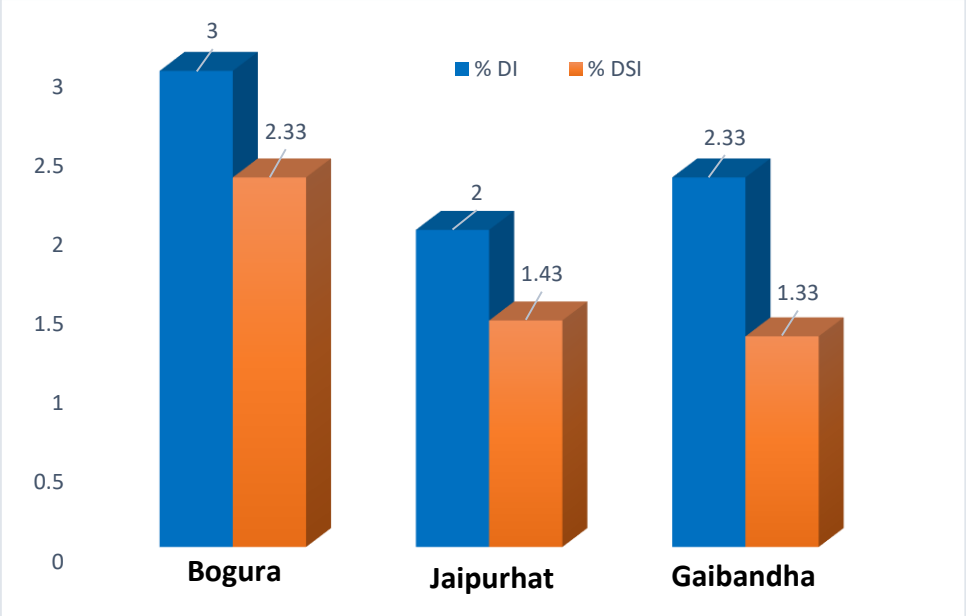


Figure 16. Disease incidence and disease severity of *PVX* in selected district

4.3. Molecular Detection

From the survey study, it was determined that *PLRV* was found to be the most predominant virus in Bangladesh. Plant viruses attack to the vascular system at phloem level. *PLRV* is found in very low concentration at phloem level. For the reason, RT-PCR is the most reliable method to detect *PLRV*.

4.3.1. RT-PCR Amplification of *PLRV-CP* Gene

For RT-PCR amplification for *PLRV-CP* gene, total RNA was extracted from naturally infected potato leaves that was collected from surveyed area. For cDNA synthesis total RNA was used. For RT-PCR amplification cDNA was subjected to use as a template. For amplification of 346bp fragment of *PLRV-CP* gene, gene specific primers (*PLRV*-346-FP/ *PLRV*-346-RP) were used and gradient PCR was done to optimize annealing temperature at 55ºC. The PCR product was analyzed on agarose gel (1%) along with ethidium bromide and amplification was measured by using 100 bp and DNA marker and the results are presented as Figure 17.

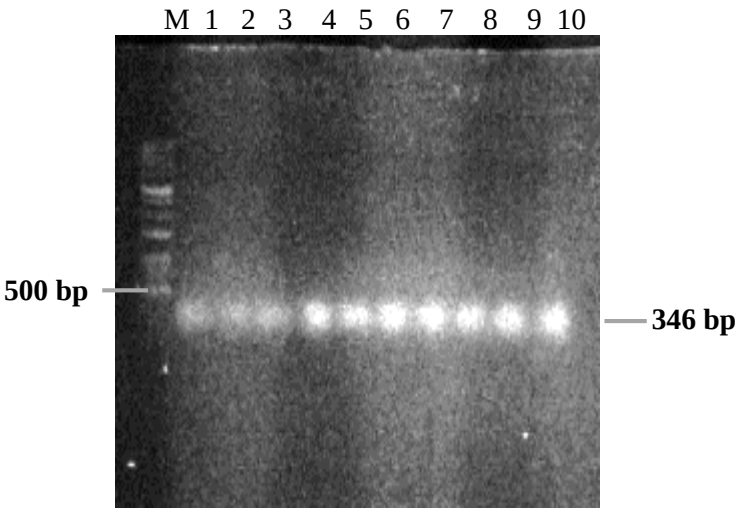


Figure 17. *PLRV-CP* gene amplification analyzed in agarose gel (1.5%) with 100bp DNA ladder

Where, M= 100bp DNA ladder,

Lane 1-10= *PLRV-CP* gene amplification at 346 fragment

DISCUSSION

Potato is considered as one of the most important crops worldwide as well as in Bangladesh. There are number of pathogens affecting potato yields such as viruses, bacteria, phytoplasma, fungi, etc. Among the pathogens, viruses are the major threat in potato production as reported by Bauding and Chatenet (1988). They also reported that potatoes are affected by more than 50 viruses and phytoplasma diseases worldwide. Bantarri *et al*., 1993 and Hossain *et al*., 2019 narrated that *PLRV, PVY,* and *PVX* are the severely affecting potato viruses that reduce potato yields by 10-90%. The incidence of potato viruses like *PLRV, PVY and PVX* are major constraint in potato production worldwide and also in Bangladesh. In this study, in three major potato growing areas in northern region of Bangladesh were surveyed and data were collected on the basis of typical symptoms of major potato viral diseases. Variety of virus related symptoms (yellowish-green mosaic, leaves malformation, stunted growth and rigidity, plant yellowing and wilting) were observed in the surveyed potato fields. Although visual inspections considered as a traditional method for identifying plant viruses in the field, many of asymptomatic plants were screened as *PLRV, PVY* and *PVX* positive plants. Consequently, this method is insufficient and not always reliable time of infection, plant variety, growth stage, virus strain and other environmental factors (Batool *et al*., 2000**)**. In addition, some *PLRV, PVY* and *PVX* strains can cause symptomless infection in potato plant (Mulder *et al*., 2008).

In the survey study, it was determined that both local and hybrid varieties of potato were cultivated by the farmers in surveyed areas. It was also observed that maximum cultivated area of potato was occupied with hybrid varieties viz. Cardinal, Diamond, Granular, Asterics, Romana, Alovery, Hira, Carriage, Destiny, Musica, Roseta, Clear, due to contribute higher yield. Although some local varieties viz. Hagrai, Sada pakri, Lal pakri, Fata pakri, Bot pakri, Pahari pakri, Jam alu, have high yield potentiality and nutritional values with very

good taste. From the survey study, it was also noticed that the source of seed tubers was varied that depend upon farmers choice. The main sources of potato seed tubers are Govt. agency (BADC), cold storage and home storage. It was also noted that farmers are conscious about the sources of potato seed tubers as well as higher yield. Farmers were used seed tuber treating agents, fungicides for seed tubers treatment and also sprayed insecticides to prevent the insect vectors like aphids. They were much conscious about the devastating disease of potato for example late blight of potato. But most of the farmers don't have ideas about potato viral diseases.

Only visual observation, on the basis of biological properties is not enough and reliable for virus detection. On the other hands, virus study on the basis of physio-chemicals properties using ultra-microscope/electron is also not visible due to highly expensive and need skill personals to maintain. Many agricultural research institutions cannot afford to have an electron microscope facility due to the prohibitively high costs involved in installation and maintenance of the facility. Viral coat protein based method such as DAS-ELISA is not reliable to detect the *PLRV* due to low viral concentration at phloem level and weekly immunogenic. RT-PCR is more reliable, robust, and highly sensitive in a short time (Schoen *et al.,* 1996). Molecular detection is the most reliable way of confirmation of any organism. Saiki *et al.,* 1985 described that successful PCR reaction is because of the choice, quality & accuracy of various factors, like template DNA/cDNA (RT-PCR), primers, dNTPs, concentration of magnesium ion, choice of polymerase enzyme and primer's annealing temperature. As a first step of this study we develop RT-PCR mediated commercial scale screening protocol of *PLRV*. As RT-PCR gives higher sensitivity with high speed diagnosis and reduced sample size, therefore, it was found good alternative to other diagnostic methods like ELISA. *PLRV* detection were made quite easy and possible by the database of nucleotide sequences for many plant pathogens like viroids, viruses, etc. The RT-PCR Similarly, a number of scientists have reported the usefulness of PCR for the detection of many plant

viruses e.g. apple scar skin, *grapevine virus A*, *pome fruit virus*, and *potato virus A* from dormant tubers (Haididi and Yang, 1990; Minafra *et al.*, 1992; Haididi *et al.*, 1993 and Singh and Singh, 1998). It has been reported that Multiplex RT-PCR was used detection of five potato viruses, i.e. *PLRV, PSTV, PVX, PVS* and *PVA* simultaneously (Nie and Singh, 2000). The procedure mentioned in this study seems cost effective and applicable. Schoen *et al.*, (1996) also suggested that, as a reliable and effective test, the RT-PCR method should be used for routine diagnosis of potato viruses because it showed higher sensitivity. In the study, *PLRV* detection procedure with RT-PCR was performed in Molecular Biology and Plant Virology Laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University. In total 27 *PLRV*+ve samples were used for molecular detection from selected upazila. Collected *PLRV+ve* potato leaves samples were used for RNA extraction, and that were then being subjected to cDNA synthesis. Before going for cDNA synthesis, RNA were confirmed in 1% agarose gel. *PLRV* virus was confirmed through RT-PCR amplification at 346 bp fragment. RT-PCR products were analyzed with 100bp DNA ladder and visualized under UV lights in gel illuminators as well as gel documentation systems.

CHAPTER V

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Potato is an important popular crop in Bangladesh and its demand is much higher than any other vegetables. It suffers from various diseases but least concrete information regarding their distribution, incidence and epidemiology is available in Bangladesh. A serious constraints to agricultural productivity and a constant pressure to global food security and hunger are infections of plants with viruses. *Potato leaf roll virus* (*PLRV*) is widely distributed with high incidence (10-90%). *PLRV* which is damaging nearly 20 million tons of the potato crops every year global. Therefore, the present study has been designed to determine the disease incidence of major potato viruses present in three selected norther districts of Bangladesh, viz. Bogura, Joypurhat and Gaibandha. Overall, three upazila (sub-stations) from each district (which were at least 20 km apart) and three spots from each upazila was selected for sample collection and disease incidence monitoring. A total of 270 samples (random/ non-random sampling) were collected on the basis of virus and viral like symptoms. Collected samples are stored at -20^{o}C and analyzed in Molecular Biology and Plant Virology Laboratory, Plant Pathology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207. The highest disease incidence of *PLRV, PVY and PVX* was identified in the surveyed fields of Bogura, Jaipurhat and Gaibandha.

Different weather components viz. temperature, relative humidity and rainfall influenced diseases incidence of potato in selected districts. Temperature in Bogura was higher than others district which is important factors for the virus multiplication and transmission through insect vectors. But application of insecticide or other chemicals was higher in Jaipurhat. Although potato tuber is the edible part, foliar application of systemic insectides may cause human health hazard. Source of seed tuber varies with farmers' choice. According to the survey, farmers choose BADC, cold storage and home storage as a source of seed tuber in the selected districts. This survey study revealed that farmers

were interested to grow the hybrid cultivars of potato for their higher production. In Bogura districts, the cultivars were viz. Asterics, Cardinal, Diamond and Granular. In Jaipurhat districts, the cultivars were Asterics, Cardinal, Granular, Musica. In Gaibandha districts, the cultivars were Asterics, Romana, Granular and Cardinal. From the local variety pakri was the most cultivated variety in all the selected districts.

The main goal of this study was to identify the major potato viruses in the selected districts and to develop a reliable RT-PCR based molecular detection method for local strain of *PLRV*. To achieve this goal, total RNA was extracted from *PLRV* positive plants and cDNA was synthesized. Specific to *PLRV-CP* gene's primers were used for RT-PCR amplification at 346bp fragments.

Finally it may be concluded that potato viruses are an upcoming threats that can cause a famine. So proper concern is needed in this perspectives and routine laboratory diagnosis need to be performed to detect the potato viruses from plant parts/seed tubers. For routine laboratory diagnosis, RT-PCR can be done to detect the potato viruses because it is more reliable, sensitive and highly robust. Moreover, this technique also needs to perform for detection of other potato viruses present in Bangladesh.

CHAPTER VI

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CHAPTER VII

APPENDICS

Appendix-I: Questionnaire of the study

TITLE OF THE PROJECT

INCIDENCE AND SEVERITY OF MAJOR POTATO VIRUSES IN SELECTED NORTHERN REGIONS OF BANGLADESH AND IT'S MOLECULAR DETECTION

"Questionnaire for potato cultivars to investigate the incidence of major potato viruses in Bangladesh''

1. Information of DAE personnel at upazila level:

   Name:…….

   Designation:……

   Address:……

   Cell No:……

2.0. Information of growers/farmers:

2.1. Name……

2.2 Gender: Male Female

2.3 Age :(Code:1 =20 or less,2=21to30,3=31 to 40,4=41to50, 5=51 or above

2.4 Educational background :(Code:1=5 to 8,2=8 to SSC, 3=HSC, 4=Graduate

2.5 Area cultivated :…….

2.6 Previous crops history of selected area (Potato cultivated or not?)

2.7 Communication with others organization: Yes/No

3.0. Information about potato seed tubers

3.1. Source of seed tubers:

   a) Home storage

   b) Cold storage

c) Improve farmer ☐

d) Govt. agencies ☐

3.2. If source of seed tubers from company then please be specific……

3.3. Information about seed tuber treatments:

a) Hot water treatment ☐

b) Treated with ash ☐

c) By using fungicides ☐

d) Other: ☐

3.4. Name of the fungicides used for seed tubers treatment:

a) MEMC ☐

b) Boric acid ☐

c) Bavistin ☐

d) Other ☐

4.0. Information about potato varieties:

4.1 Name of the potato varieties used:

a) Cardinal. ☐

b) Diamond ☐

c) Asterics ☐

d) Lal pakri. ☐

f) Granular ☐

g) Romana ☐

h) Other ☐

4.2. Mostly cultivated varieties:

a) Local ☐

b) Improve varieties ☐

c) Hybrids ☐

4.3 Date of sowing

5.0. Information about disease incidence of major potato viruses:

5.1. Major potato viruses symptoms that appear at investigated area:

a) PLRV ☐

b) PVY ☐

c) PVX ☐

d) other ☐

5.2. Incidence of *PLRV* on the basis of typical morphological symptoms:

a) Severe / Epidemic form ☐

b) Moderate ☐

c) Low infection ☐

d) No-infection ☐

5.3 Incidence of *PVY*

a) Severe / Epidemic form ☐

b) Moderate ☐

c) Low infection ☐

d) No-infection ☐

5.4 Incidence of *PVX*

a) Severe / Epidemic form ☐

b) Moderate ☐

c) Low infection ☐

d) No-infection ☐

5.1. Status of insect vectors:

a) Several numbers ☐

a) Moderate numbers ☐
b) Few numbers ☐
c) No insect vectors present ☐

5.0. Used of insecticides to control the insect vectors:

a) Epidachloromite ☐
b) Monochotophos ☐
c) Dimethoate ☐
d) Pirimicarb ☐
e) Pymetrozine ☐
f) Others. ☐

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Signature of Farmer/Grower Signature of Research Assistant

Signature of Research Supervisor

Appendix-II: Disease Incidence (DI) and Disease Severity (DS) of Surveyed Area in 2019-2020

Sl No	Districts	UpaZila	*PLRV*		*PV X*		*PV Y*	
			% DI	% DSI	% DI	% DSI	% DI	% DSI
1	Bogura	Shibgonj	29	23	1.5	0.5	17	15
		Shajahanpur	**33**	**28**	3	2.5	21	17
		Sadar	30	26	3	3	23	18
2	**Jaipurhat**	Kalai	20	15	2.5	2	13	7.7
		Khetlal	23	19	1	0.4	16	10
		Sadar	26	17	3	1.9	16	9
3	**Gaibandha**	Gobindogonj	22	17	1.5	0.5	16	13
		Polashbari	25	20	2	1.5	20	14
		Sadar	23	19	3.5	2	18	12

Apendix-III: Anova Table

District	Diseases	% DI	% DSI
Bogura	*PLRV*	30.667A	25.667 A
	PV Y	20.333 B	16.667 B
	PV X	2.500 C	2.333 C
CV (%)	_	7.24	7.34
LSD	0.05		
Jaipurhat	*PLRV*	23.000 A	17.000 A
	PV Y	15.333 B	8.900 B
	PV X	2.333 C	1.433 C
CV (%)	_	10.72	7.76
LSD	0.05		
Gaibandha	*PLRV*	23.333 A	18.667 A
	PV Y	18.000 B	16.000 B
	PV X	2.333 C	1.333 C
CV (%)	_	7.77	8.7
LSD	0.05		

Appendix-IV: Stock Solution

Forward primer: Forward primer + 532 µl DEPC treated water

Reverse primer: Reverse primer + 392 µl DEPC treated water

Appendix-V: Working Solution

10µl Forward primer + 90 µl DEPC treated water =100 µl (10 pmoles)

10µl Reverse primer + 90 µl DEPC treated water =100 µl (10 pmoles)

Appendix-VI: cDNA Synthesis

RNA-4 µl

Forward/ Reverse primer -1 µl

Nucleus free water-6 µl

Appendix-VII: 100 ml Gel Preparation (1% gel)

1x TBE Buffer-100ml

Agarose gel-1000 mg

Heat for 3 minutes

After cooling, Ethidium bromide- 2.31 µl

Appendix-VIII: 6X DNA Loading Dye

Bromophenol blue - 20 µl

DEPC treated water - 60 µl

Appendix-IX: Gel Electrophoresis

Sample-2 µl

Loading Dye-2 µl

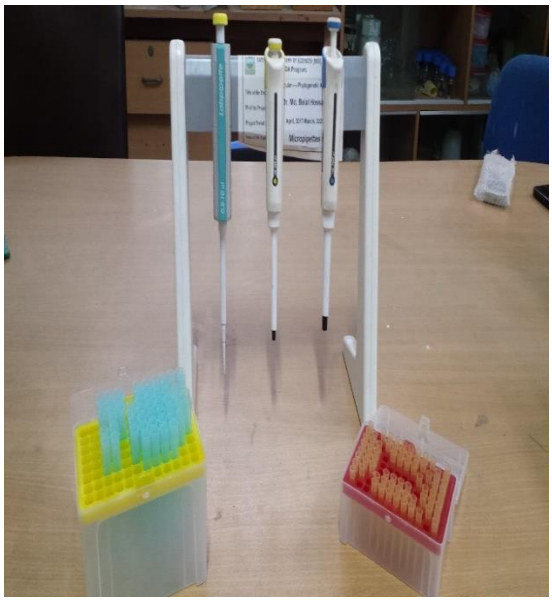
Appendix-X: Data collection during survey

Sample collection

Data collection

Appendix-XI: Laboratory equipment

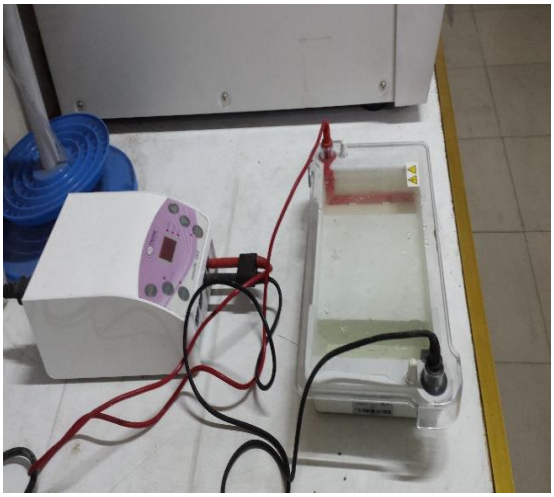
Magnetic stirrer



Micro peppetes

Refrigerate centriguge

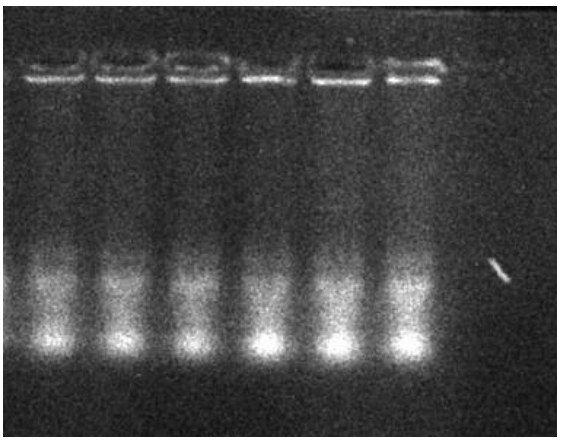
PCR machine



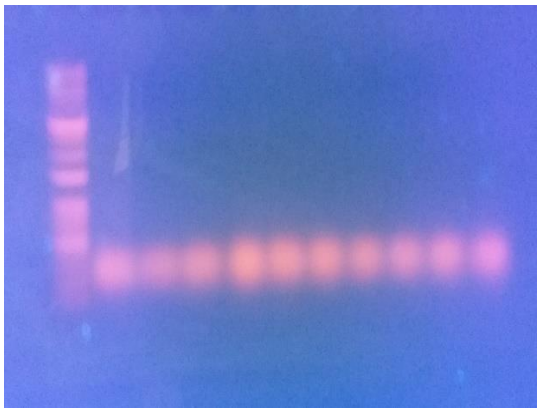
Gel tank with power

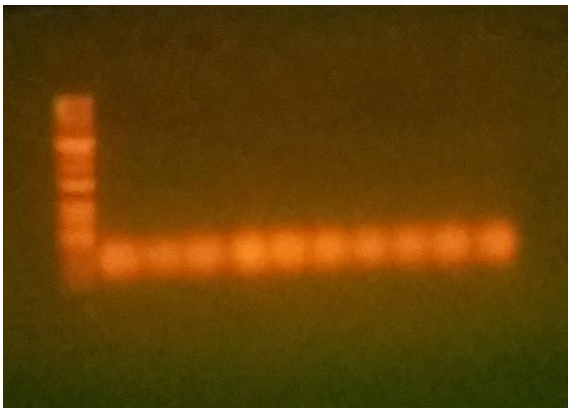
Vortex Shaker

Appendix-XII : Gel run



RNA confirmation





PCR product in 1% gel