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Occurrence and Incidence of Major Potato Viruses in Bangladesh and their Molecular Detection

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Abstract

Incidence of major potato viruses in eight major potato growing areas of Bangladesh, viz. Panchagarh, Thakurgaon, Nilphamari, Rangpur, Bogra, Chapainawabgonj, Pabna and Munshiganj were investigated in field samples. Overall, three upazilla (sub-districts) from each district (which were at least 20 km apart) were selected for samples collection and disease incidence monitoring. A total of 240 samples were collected on the basis of virus and viral like symptoms. Collected samples were storage at 40C and analyzed in Molecular Biology and Plant Virology lab, Central Research Laboratory of Sher-e-Bangla Agricultural University (SAU), Dhaka. Enzyme Linked Immunosorbent Assay (ELISA) tests revealed that Potato leaf roll virus (PLRV) was most predominant virus followed by Potato virus Y (PVY) and Potato virus X (PVX). During the year 2017-18, the relative frequency of infection by PLRV and PVY was 31 and 2% of infected samples respectively. Single, double and triple infections were 34, 45 and 2.0% respectively. Infection of detected potato viruses in all investigated districts with different percentage was almost similar. Major potato viruses' symptoms that appear at investigated areas are PLRV, PVY and PVX and their relative incidences level in random samples were severe to moderate. In all investigated areas, PLRV and PVY appeared in severe to moderate level and their % incidence was (18 & 41%) and (3 & 17%) respectively, while the PVX was appeared in moderate level and % incidence of PVX 18%. In this study, the sources of potato seed tubers was also studied and observed that in most of the cases farmers of selected areas are using continuously same field and used their own seed tubers that was kept in cold storage condition and % frequency was 67.6%. PLRV and PVY were also detected via reverse transcriptase polymerase chain reaction (RT-PCR). A 346 bp and 480 bp amplicon of PLRV and PVY- coat protein (CP) gene was amplified and the nucleotide sequences of amplified.

Keywords: Potato; *PLRV; PVY; PVX*; ELISA; RT-PCR; Bangladesh.

1. Introduction

The potato (*Solanum tuberosum L.*) is major vegetable crop in Bangladesh as well as world's leading staple food crop in many countries. Among the most important food crops in the world, Potato is ranked fourth in production after rice, wheat and maize [1-3]. The potato crop gives 12-15 times more yield per hectare and calories production per unit area is higher than wheat, maize and rice. The potato tuber is an excellent source of carbohydrates, protein and vitamins [4-6]**.** In the world, potato is cultivated 19,098,300.00 ha of lands and production 381,682,000.00 tons FAOSTAT [7] and in Bangladesh, cultivated 4, 75,488 ha of lands and production 94, 74,098 metric tons [8]. Potato is cultivated twenty three major growing areas in Bangladesh. In comparison to other agricultural crops, the seed cost of potato cultivation is much higher. Bangladesh Agricultural Development Corporation (BADC) reported that the seed cost of potato is liable to 30 - 40% of total production cost [9]. Bangladesh imported high yielding foreign potato varieties at the cost of above US $ 150.00 per quintal which engross large amount of foreign currency every year [10]. Potatoes are infected a biotic and biotic factors. Due to abiotic factor such as hail, floods and droughts, and to temperature-related events such as frost and heat waves etc. are cause 25% yield losses. Due to biotic factor such as powdery scab whereas the insects tuber moths, leaf miners and aphids were reported 50% yield loss. 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, PVX* and *PVM* which have been reported in Bangladesh and causes 10-90% yield losses. More than 35 different viruses are known to affect potatoes. Currently, no reports are available

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about high yielding commercial varieties or advance potato lines in Bangladesh has shown durable resistance against viruses.

According to the Viruses in categories 1, 2 and some in category 3 can be considered the most economically important in potato production worldwide. Yield reduction by these viruses is usually higher than 50% in most susceptible cultivars. So we needed to determine the potato viruses through reliable detection methods. 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 [11]. 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. Within the main scope of revealing the knowledge about the sanitary status of plants and presence of potato viruses in the country of Bangladesh, this study was aimed to detect the major potato viruses (like *PLRV, PVY, PVX*) based on viral coat protein through serological test (ELISA) and based on nucleic acid through RT-PCR. The ultimate target of this study was to provide a platform for plant viruses' diagnostic research that will be helpful to create proper management approaches to manage or control plant viral diseases in Bangladesh.

2. Materials and Methods

The study was conducted to investigate the distribution and incidence level of major potato viruses in commercial growing areas of Bangladesh and their molecular detection. In total three experiments were performed throughout the study period in order to investigate the major potato viral diseases. The experiments are; Experiment-I: Collection of disease samples from major potato growing areas of Bangladesh, Experiment-II: Determination of disease incidence (DI) of major potato viruses. Experiment-III: Detection of identified potato viruses through ELISA and RT-PCR test.

2.1. Experiment-I: Collection of Disease Samples From Major Potato Growing Areas of Bangladesh

2.1.1. Surveys of Major Potato Growing Areas of Bangladesh

During the year 2016-17 and 2017-18, a survey was conducted in eight major potato growing areas of Bangladesh viz Panchagarh, Thakurgaon, Nilphamari, Rangpur, Bogra, Chapainawabgonj, Pabna and Munshiganj. Overall, three upazilla (sub-districts) from each district (which were at least 20 km apart) were selected for samples collection and disease incidence monitoring. Basic information regarding potato crop viz. varieties, sources of seed tubers, irrigation, agricultural practices and disease incidence etc. was also collected from farmers/growers of all areas through a structured questionnaire.

2.1.2. Samplings

Virus infected leaves/tubers samples were collected. At least 15 random and 15 non-random samples were collected from each upazilla (sub-districts) in farmer's field by moving diagonally in the field. A total of 240 samples of 6-8 weeks old field growing potato plants were collected on the basis of virus and viral like symptoms. A single sample was consisted of three single leaflets taken from top, middle and bottom, and placed in polythene sample bag and stored in an ice box. Samples were labeled appropriately to indicate location, sample number and date of collection. These samples were brought to Molecular Biology and Plant Virology lab, Central Research Laboratory, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207. All samples were stored at 4°C.

2.1.3. Observation of the Symptoms

Symptoms of the diseases were studied by visual observation. Sometimes hand lens was used for critical observation of the disease and sometimes a disease was identified based on matching the observed symptoms in the infected leaves and tuber with the symptoms. Identification of all the virus diseases was finally confirmed by identification of the associated virus organism through isolation.

2.1.4. Collection of Diseased Specimen and Processing

Potato tubers were collected from infected disease plant. The specimens were preserved in the laboratory room following standard procedure of preservation. The di`sease specimens were arranged in district wise and preserved until the protein and RNA extraction was made.

2.2. Experiment-II: Determination of Disease Incidence (DI) of Major Potato Viruses

2.2.1. Data Collection During Survey

During the survey in the selected study areas, total numbers of infected plant and tuber were counted. Primary identification disease was done by visual observation. Infected area on leafs was also counted by visual observation.

2.2.2. Determination of Disease Incidence

Data were collected from farmer's field during potato cultivation to observe disease incidence in potato. Randomly and Non-random 544 m^2 (435m × 5m)/4 area was selected in farmers field. Total leaves and tuber were counted from 544 m^2 selected area and among them disease infested leaves and tubers were counted to calculate

percent disease incidence. All data were collected from 8 district 24 upozila selected locations followed by a prepared simple questionnaire.

2.2.3. Data Analysis

The collected data was arranged then put in the excel sheet and analysis by using the computer basis software SPSS.

2.3. Experiment-III: Detection of Identified Potato Viruses Through ELISA and RT-PCR Test

2.3.1. ELISA Tests

2.3.1.1. Serological Detection/ELISA Test

In total 32 samples out of 240 samples were tested against three major potato infecting viruses viz. *PLRV*, *PVY* and *PVX* in polystyrene microtitre plate through standard Double Antibody Sandwich (DAS) ELISA as described by Clark and Adams [12] using antibodies (BIOREBA AG kit) and enzyme substrate. Positive results of DAS-ELISA were judged based on the readings of the ELISA reader (EPSON LX-300) at 405 nm compared with the cut off value supplied by the data sheet of the kit, in addition to the development of yellow color in the wells of ELISA plates. A sample is considered as virus infected when the absorbance of 405 nm was at least thrice of that healthy control. The relative occurrence or % incidence of *PLRV* or *PVY* or *PVX* was determined by applying following proportionate formula [13].

$$\text{Incidence (\%)} = \frac{\text{Number of samples positive for a Virus}}{\text{Total number of samples tested}} \times 100$$

2.3.2. Primer Designing

Primers were designed by using primer-3 latest version software. During the primers selection multiple alignments were done by using Clustal-W latest version software for identification the conserved regions. The following primers pair was used in amplification of *PLRV* and *PVY* CP-gene.

Table-1. Primer Sequences used in amplification of *PLRV* and *PVY* CP-gene respectively.

Primer ID	Sequence (5'-3')	PCR product size(bp)
PLRV346-FP	CAGGCGCCGAAGACGCAGAA	346
PLRV346-RP	TTTGGCGCCGCCCTTCGTAA	
PVY480-FP	ACGTCCAAAATGAGAATGC	480
PVY480-RP	TGGTGTTCGTGATGTGACCT	

2.3.3. Extraction of RNA from PLRV and PVY +ve leaves samples

Total RNA was extracted from *PLRV* and *PVY* +ve-experimental host plant leaves that maintained in the net house, by the "single step" method described by Chrzanowska [14]. Leaves were collected in 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 01 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 13,000 rpm at 4°C for 15 minutes was given. The RNA rich upper portion of the centrifuged solution was poured into another Eppendorf. 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 13,000 rpm at 4°C. Washing of pellet was done by adding 10 ml ethanol (75%) and supernatant was removed. The sample was re-suspended properly through pipetting and centrifuged at 10,000 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 (20 µl) was used to re-suspend RNA and put at -70°C. After quantification through spectrophotometer RNA quality was confirmed 1% agarose gel.

2.3.4. cDNA Synthesis

"First Strand cDNA Synthesis kit" (Ferments) was used to synthesize cDNA. The cDNA was synthesized by adding the total RNA @ 1µg plus 1.0µl of the reverse primeri.e.10 pmoles plus nuclease-free water to make final volume at 11µl and mixed slightly and spun gently in a micro-centrifuge for 3-5 seconds. After, giving 5 minutes of incubation at 70°C plus ice chillingplus addition 05X reaction buffer 4µl plus Ribonuclease-inhibitor 1µl (20 u/µl) and 2µl of 10mM dNTPs mix 02µl (20u/µl) then incubation at 37°C, 5 minutes and reverse transcriptase 02µl (M-MuLV 20u/µl) for volume of 20 µl reaction. Lastly, incubation at 37°C plus 60 minutes incubation time before stopping the reaction and ice chilling at once. cDNA will be synthesis from the extracted RNA following the standard protocol.

2.3.5. RT-PCR Amplification

2.3.5.1. Amplification of 346 bp Fragment of PLRV- CP Gene

RT-PCR amplification was performed by using the designed primers and cDNA was used as template of PCR reaction. PCR amplification was performed by 35 cycles after initial denaturation at 94ºC for 4 minutes in a thermal cycler starting with denaturation at 94ºC for 1 min, primer annealing at 55ºC for 1 min and extension at 72ºC for 1 min followed by final extension at 72ºC for 10 minutes.

2.3.5.2. Amplification of 480 bp Fragment of PVY-CP-gene

PCR reaction was performed using 3µl of the first strand cDNA as template, 2µl (10 pmol) of each forward and reverse primers, 2µl of 1mM dNTP mixture, 2 µl of 10X reaction buffer (20mM Tris HCl, pH 8.4, 50mM KCl), 2U of Taq polymerase and made 20 µl final volume of reaction mixture with PCR water. PCR amplification was performed by 35 cycles after initial denaturation at 94ºC for 4 minutes in a thermal cycler starting with denaturation at 94ºC for 45 sec. primer annealing at 55ºC for 45 sec. and extension at 72ºC for 45sec. followed by final extension at 72ºC for 10 minutes.

2.3.5.3. Agarose Gel Electrophoresis and gel elution

Amplified fragments of *PLRV* and PVY-CP were separated on 1% agarose gel, stained with (0.5-1µg/ml) ethidium bromide and analyzed under UV light. The amplified fragments were cut for elution. Elution was done by using DNA Gel Extraction kit.

3. Results

3.1. Symptomology

Stunting, systemic vein clearing, mosaic, mottling, curling, shortening of leaves, dark green and vein-banding were observed on 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. In some plants with mosaic symptoms, mottling, shortening of leaves, dark green and stunting infected plants were found. In few plants with systemic vein clearing and vein-banding were found (Figure 1). In case of *PLRV*, infected tuber showed phloem necrosis (Figure 2). Symptomology is not a reliable method for confirmation of viruses but it is an initial step to disease diagnosis. For further confirmation, collected samples were preceded for serological detection through ELISA test and molecular detection through RT-PCR amplification.

Figure-1. Typical symptoms of *PLRV, PVY and PVX* compere with normal leaflet.

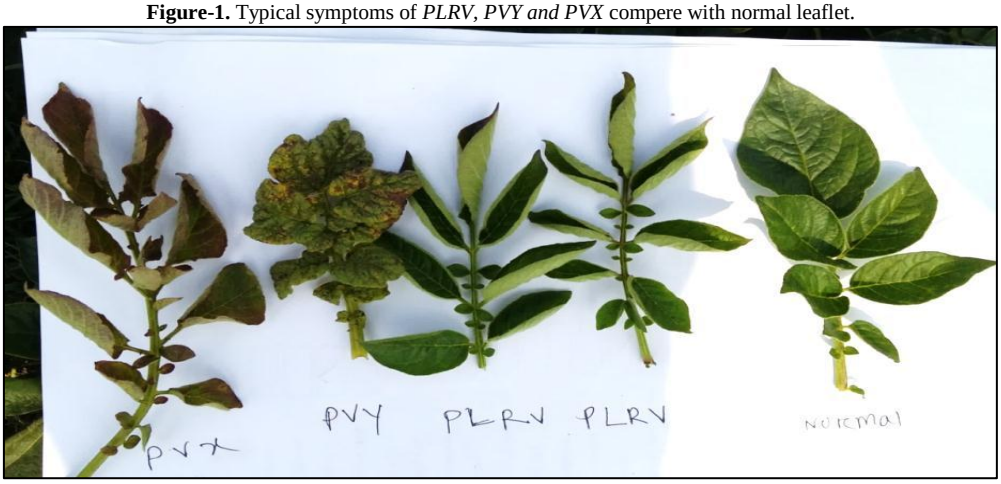
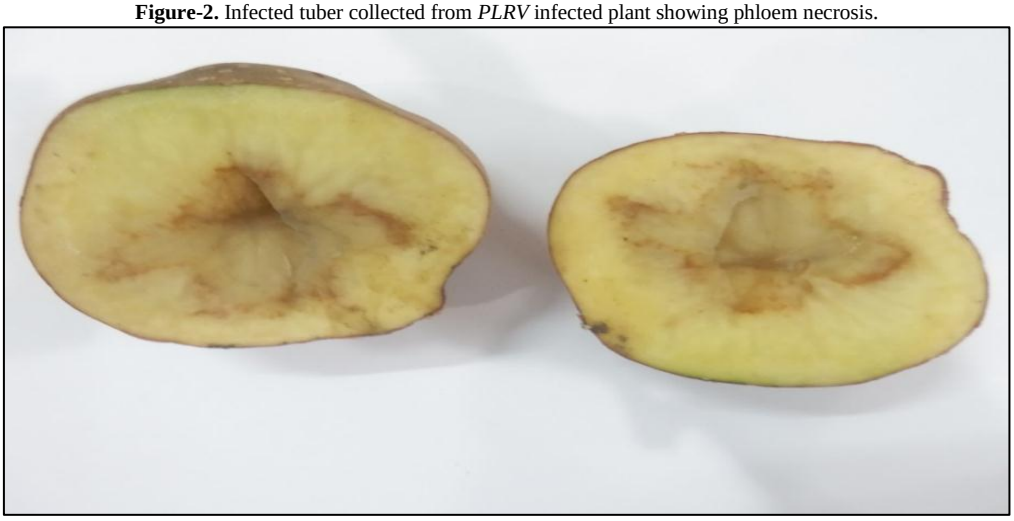


Figure-2. Infected tuber collected from *PLRV* infected plant showing phloem necrosis.



3.2. Serological Detection

In total 32 samples out of 240 samples were tested against three major potato infecting viruses. *PLRV* was most predominant in all locations and other remaining viruses varied in their order of frequency (Table 2). Yellow colour indicates that there was positive reaction with virus antigen using monoclonal antibodies of *PLRV*, *PVY* and *PVX* . The relative frequency of infection by *PLRV and PVY* was 31 and 2% of infected samples respectively. Single, double and triple infections were 34, 45 and 2.0% respectively (Figure 3). Infection of detected potato viruses in all investigated districts with different percentage is almost similar as clearly shown in figure 4.

Figure-3. Frequency of single, double and triple infections of *PLRV*, PVY and *PVX*

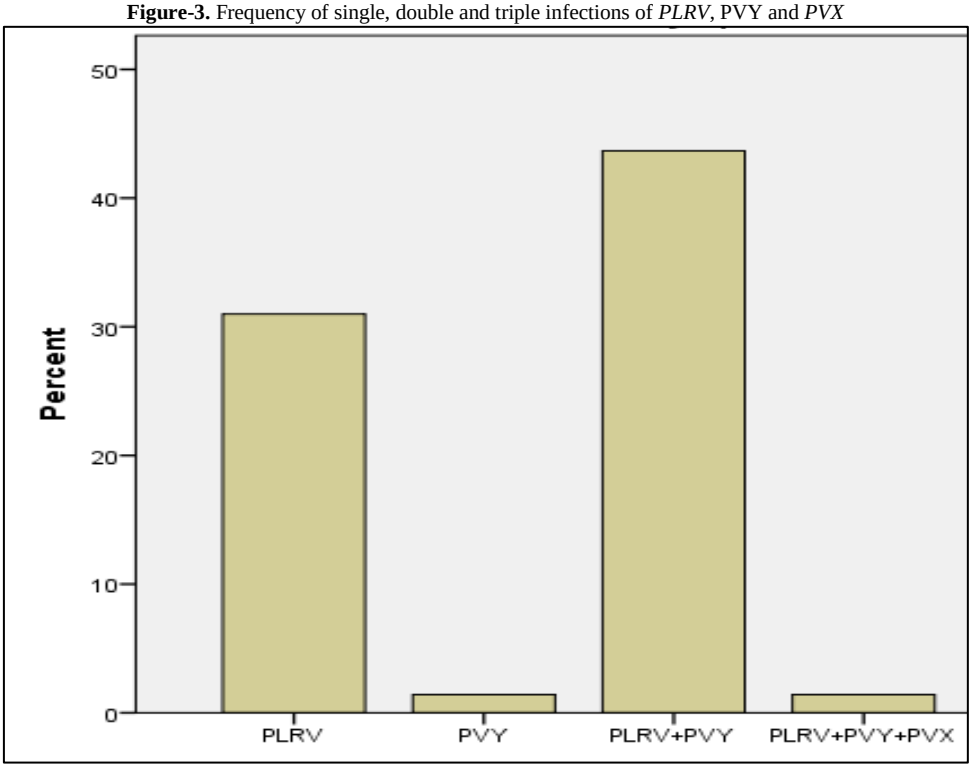
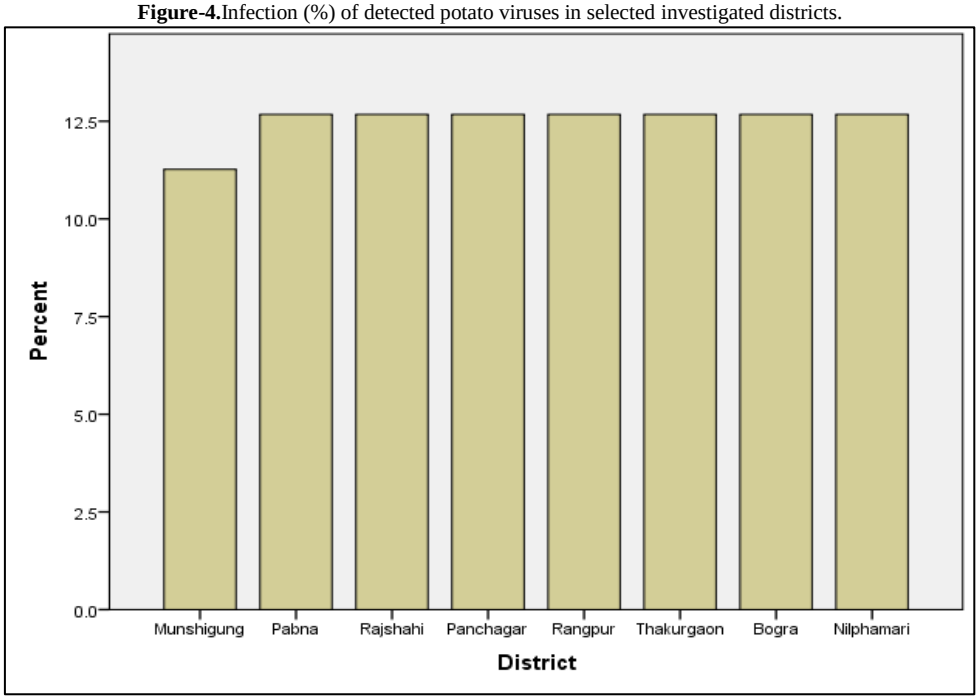


Figure-4. Infection (%) of detected potato viruses in selected investigated districts.



3.3. Incidence (%) of PLRV, PVY and PVX

Major potato viruses' symptoms that appear at investigated areas are *PLRV*, *PVY* and *PVX* and their relative incidences level in random samples were severe to moderate. In all investigated areas, *PLRV* and *PVY* appeared in severe to moderate level and their % incidence was (18 & 41%) and (3 & 17%) respectively. While the *PVX* was appeared in moderate level and % incidence of *PVX* 18% as shown in the figure 5. In this study, the sources of potato seed tubers was also studied and observed that in most of the cases farmers of selected areas are using continuously same field and used their own seed tubers that was kept in cold storage condition and % frequency was 67.6%. The second source of potato seed tubers was government organization and % frequency was 15.5%. Others

sources of potato seed tubers were local market, home storage, company seed, NGO supply and their % frequency was 8.5, 5.6, 1.4, 1.4% respectively (Table 2). Due to continuous using of same field and own seed tubers virus is accumulating in the field and this is the major factor in the disease development and spread.

Figure-5. Incidence of *PLRV, PVY* and *PVX* in different growing stages

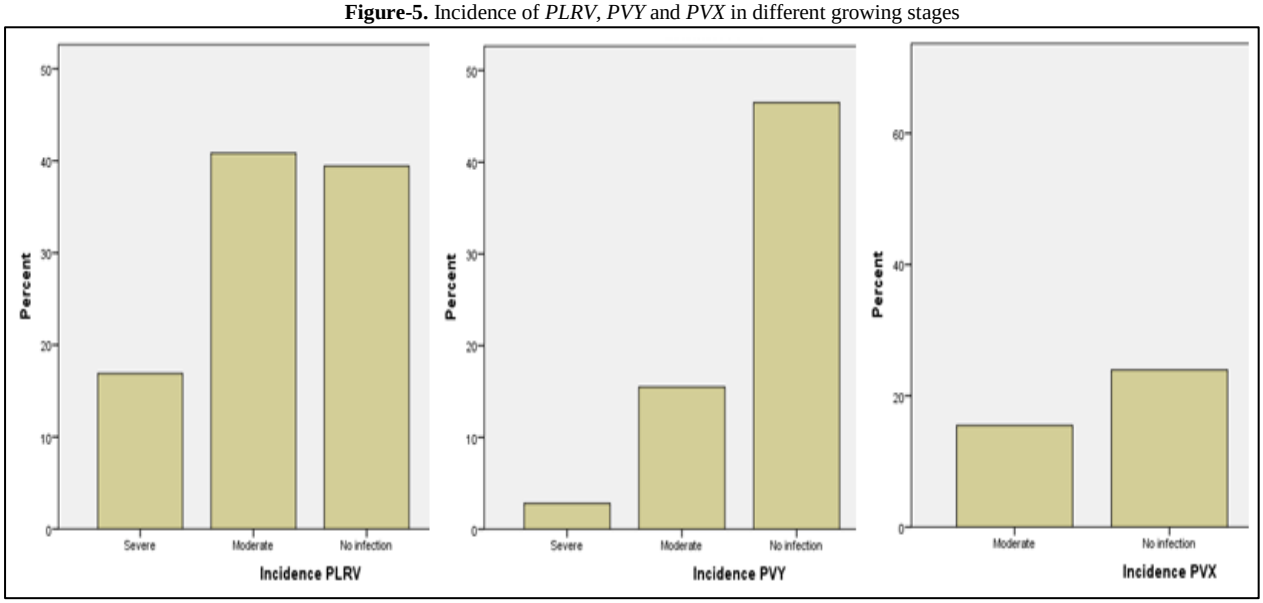


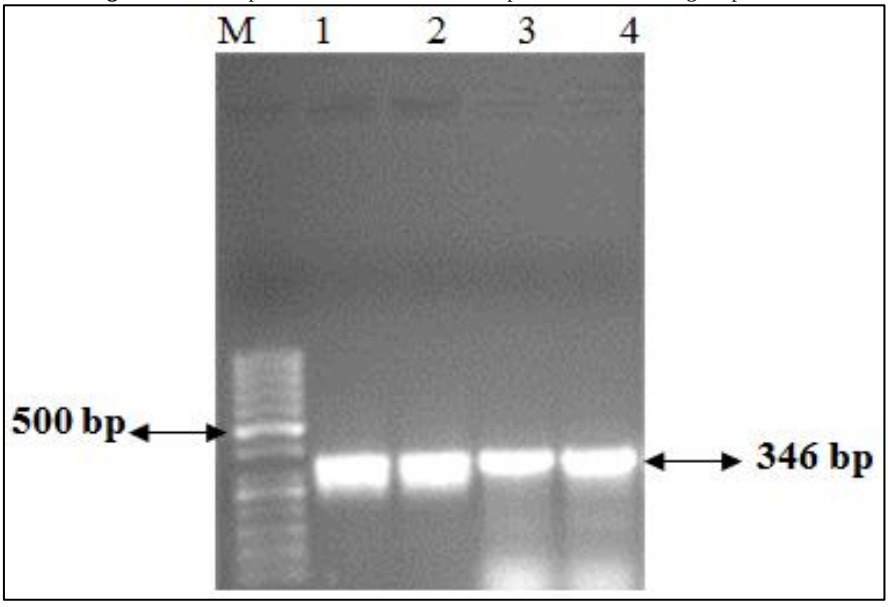
Table-2. Source of potato tubers used in potato cultivation in 2015-2016 at selected areas

Sources	Frequency	Percent
Home Storage	4	5.6
Cold Storage	48	67.6
Local Market	6	8.5
Government Organization	11	15.5
Company Seed	1	1.4
NGO Supply	1	1.4
Total	71	100.0

3.4. Molecular Detection of PLRV-CP gene via RT-PCR

PLRV infected potato plants were selected on the basis of typical symptoms and stand upright rolled leaves were used for RNA extraction. Total RNA was extracted form leaves and cDNA was synthesized from extracted RNA, that was used as a template in PCR amplification, using gene specific primers, designed to amplify 346 bp fragment of *PLRV* from the coat protein gene region. Total volumes of RT-PCR products were resolved on 1.5% agarose gel along with 50 bp molecular weight marker. The expected size of the PCR product was ~ 346 bp. Amplification was sharp and clear as depicted in figure 6.

Figure-6. PCR amplification, cDNA used as template with *PLRV346* gene primers.

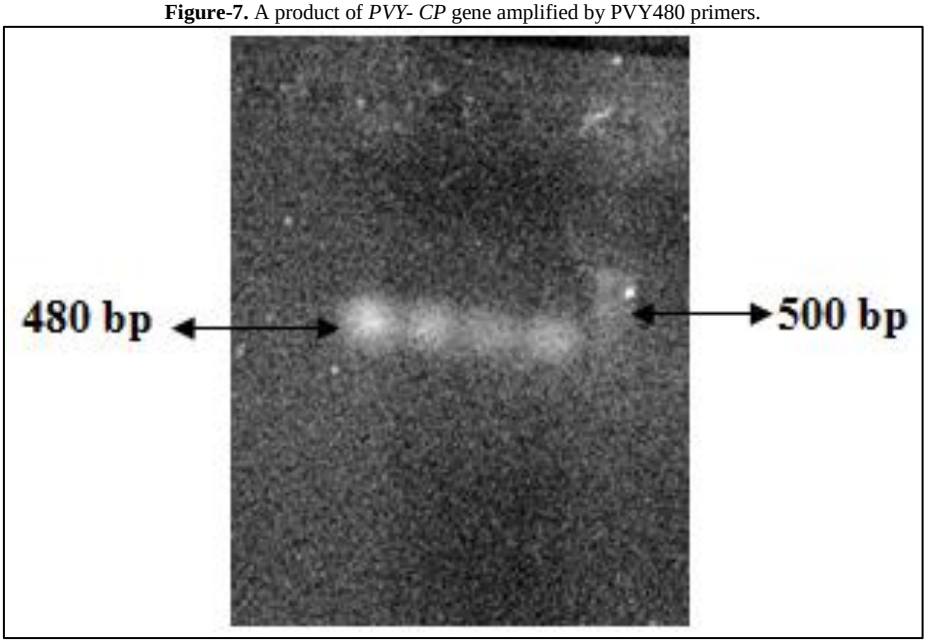


M= 50bp DNA Ladder and
Lane 1-4 PCR amplified at 346 bp

3.5. Amplification of CP-PVY gene

PVY infected potato plants were selected on the basis of typical symptoms and severe mosaic appearing leaves were used for RNA extraction. Total RNA was extracted form leaves of the and cDNA was synthesized from extracted RNA that was used as a template in PCR amplification, using gene specific primers, designed to amplify 480 bp fragment of *PVY* from the coat protein gene region. Total volumes of RT-PCR products were resolved on 1.5% agarose gel along with HindIII molecular weight marker. The expected size of the PCR product was ~ 480 bp. Amplification was sharp and clear as depicted in figure 7.

Figure-7. A product of *PVY- CP* gene amplified by PVY480 primers.



M= 100bp DNA Ladder and Lane 1-4 PCR amplified at 480 bp

4. Discussion

Potato is considered as one of the most important crops worldwide as well as in Bangladesh. 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 total eight major potato growing areas of Bangladesh were surveyed and data were collected on the basis of typical symptoms of major potato viral diseases [15].Variety of virus related symptoms (yellowish-green mosaic, leaves malformation, stunted growth, 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.

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.

Consequently this method is insufficient and not always reliable because the development of symptoms usually depends on several factors such as time of infection, plant variety, growth stage, virus strain and other environmental factors [16]. In addition, some *PLRV*, *PVY* and *PVX* strains can cause symptomless infection in potato plant [17, 18]. Moreover, not all plants that showed virus-like symptoms tested virus positive with DAS-ELISA, this might be due to the fact that some plants could exhibit virus-like symptoms as a result of unfavorable conditions. It is also possible that they were infected by other types of plant viruses which cause the same effect on potatoes [19]. For that reasons, all collected samples were screened by using molecular tests, like RT-PCR and serological tests (DAS-ELISA) are used to diagnose major potato viruses. This technique successfully detected the virus from potato leaf samples even in the leaves that were asymptomatic. These illustrates were in agreement with previous findings that to detect *PLRV* and *PVY* 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. DAS-ELISA was a reliable and sensitive method for primary screening of plant viruses [20]. Mechanical inoculation for a number of ELISA highly positive samples was conducted to confirm the virus occurrence results and to observe its related symptoms.

There was a significant difference in the infection percent among the different locations. The highest infection percentage was in the locations that belong to Pabna, Rajshahi, Panchagar, Rangpur, Thakurgaon, Bogra and Nilphamari. While in other locations such as Munshigung low infection was detected in the screened locations. In fact many factors play a crucial role in this variation or could be the causative agent of this difference in *PLRV*, *PVY* and *PVX* incidence among different locations. Of these, cultivation history, cultivation practices, preventive measurements and temperature are the main ones. The highest incidence of *PLRV*, *PVY* and *PVX* was detected in the surveyed fields of Pabna, Rajshahi, Panchagar, Rangpur, Thakurgaon, Bogra and Nilphamari. According to the

owners of these fields the source of potato seeds were home storage, local market, Government organization, private/company seed and NGO supply. The best performance showed cold storage (frequency 48%) and government organization (frequency 11%). Moreover, these samples were the only samples that collected during the fall growing season of 2015-16, and the temperature in this season is very suitable for the virus activity and multiplication. It has been noticed that there was a difference in the cultivation practices among these locations. In addition to the difference in weather condition among the selected locations, as the temperature was higher in Munsigong than others district which is important factors for the virus transmission through the insect vectors. This may indicate that the prevalence of potato viruses is not consistent from one location to another. Thus, it would be useful to perform further studies on potato viruses' prevalence in the main growing regions of potatoes in Bangladesh. The obtained information will provide a clear view of areas with the least potato viruses' prevalence which will be suitable for potato cultivation. Such information would also be useful in developing new strategies for control of viruses occurring in main potato growing regions .

5. Conclusion

In this investigation, considering the incidence level of major potato viruses in the field conditions it may be concluded that three (3) major potato viruses viz. *Potato leaf roll virus (PLRV), Potato virus Y (PVY)* and *Potato virus X (PVX)* was common in all major potato growing areas of Bangladesh. From this field survey it was also revealed that *PLRV* was most predominant virus in all selected investigation locations followed by *PVY* and *PVX*. Same results were also found when collected field samples were tested through ELISA and RT-PCR. However, such types of investigation/survey program need to carry out for consecutive years in all over the country or at least 23 districts where potato is commercially cultivated to justify the present findings. Finally it may be concluded that for routine laboratory diagnosis to detect the potato viruses from plant parts/seed tubers, various molecular methods (like RT-PCR) can be used because is reliable, robust, and highly sensitive in a short time. Moreover, in this study only two viruses (*PLRV & PVY*) were detected through molecular technique (RT-PCR), this technique also needs to perform for detection of other existing potato viruses in Bangladesh against the local strains.

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