Molecular Characterization, PCR-based Cloning and Sequencing of Coat-Protein Gene of a Bangladeshi *Potato Leaf Roll Virus* (*PLRV*) isolate and its Phylogenetic analysis

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Presented

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CERTIFICATE

This is to certify that the thesis entitled**Molecular Characterization, PCR-based Cloning and Sequencing of Coat-Protein Gene of a Bangladeshi *Potato Leaf Roll Virus* (*PLRV*) isolate and its Phylogenetic analysis** submitted to theDepartment of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology, embodies the results of a piece of bonafide research work carried out by **Registration No. 12-04856** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

Dated: 20/10/2019
Bangladesh

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Dedicated to

MyBeloved Parents

And

The Farmers

who feed the natio

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The Author

MOLECULAR CHARACTERIZATION, PCR-BASED CLONING AND SEQUENCING OF COAT-PROTEIN GENE OF A BANGLADESHI *POTATO LEAF ROLL VIRUS* (*PLRV*) ISOLATE AND ITS PHYLOGENETIC ANALYSIS

ABSTRACT

An 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-March, 2018.Total RNA was extracted from *Potato leaf roll virus* (*PLRV*) positive leaves and complementary DNA (cDNA) were synthesized from total RNA. Reverse transcriptase polymerase chain reaction (RT-PCR) based detection conditions were optimized by using coat protein (*CP*) gene specific primers. A 346 bpamplicon of *PLRV- coat protein* (CP) gene was amplified. In PCR amplification cDNAwas used as a template and for nucleotide sequencing PCR product was used as a template. Expected nucleotide sequence of amplified *PLRV-CP* gene showed 85 to 98% homology when compared to the sequences already reported in GenBank database. This explored novel *PLRV-CP* gene was characterized as a*PLRV* Bangladeshi isolate. Phylogenetic analysis was also carried out and the tree was made by using MEGA 4.0.

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List of Abbreviations

bp	Base pair
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide Triphosphate
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
EDTA	EthylinediamineTetraacetic acid
MEGA	**Molecular Evolutionary GeneticsAnalysis**
mRNA	Messenger RNA
PCR	Polymerase Chain Reaction
PLRV	Potato Leaf Roll Virus
PLRV-CP	*PLRV*-Coat Protein
RNA	Ribonucleic acid
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TAE	Tris Acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris EDTA

INTRODUCTION

The genus Solanum, to which cultivated potato belongs, is large consisting of about 1000 species. More than 200 species of potato have been found, but just eight are cultivated and have been cultivated for the last 2000 years (Smith, 1977). Among the most important food crops in the world, Potato (*Solanumtuberosum* L.) is in the fourth position next to wheat, rice and maize(Rauscher, *et al.*, 2006, Islam, *et al*., 2014 and Abbas *et al*., 2014). The potato tuber is an excellent source of carbohydrates, protein and vitamins (MacGillivary, 1953). In the world, potato is cultivated 19,098,300.00 ha of lands and production 381,682,000.00 tons (FAOSTAT, *et al*., 2017) and in Bangladesh, cultivated 4,75,488ha of lands and production 94,74,098 metric tons (BBS 2016-17).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 (Anon, *et al*., 2005).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 (Anon, *et al*., 2005).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. 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. applescar skin, grapevine virus A, pome fruit virus, and potato virus A from dormant tubers (Singh and Singh, 1998). The goal of this study is to detect and characterize *PLRV* through RT-PCR amplification, cloning and sequencing of *CP* gene for characterization of *PLRV* Bangladeshi isolate. Through this study, a reliable RT-PCR based molecular detection method was developed for local strain of *PLRV,* and a Bangladeshi isolate of *PLRV* would be found on the basis of nucleotide sequencing from cloned *CP* gene. The

sequence identity analysis of the cloned *PLRV -CP* gene will be used to assess homologies among several *PLRV* isolates reported in Gene bank database.

Objectives

The specific objectives of the proposed study are as follows:

-To develop a reliable RT-PCR based molecular detection method for localstrain of *PLRV.*

-To explore and assess homologies among several *PLRV* isolates reported in Gene Bank database.

-To characterize of *PLRV* strains as a Bangladeshi isolate on the basis of nucleotide sequencing.

REVIEW OF LITERATURE

Potato (*Solanumtuberosum* L) is growing as a leading staple crop in Bangladesh. Among vegetable affecting virus, Potato leaf roll virus (*PLRV*) is one of the top viruses and enjoys a promising position among the pathological constrains in the potato crop. It is getting alarming position with significant losses in Bangladesh. A lot of research work has been done on various aspect of *PLRV* in Bangladesh and abroad and is reviewed as under:

2.1. Potato

Potato (*Solanumtuberosum*) is an herbaceous annual plant which belongs to the Solanaceae family of flowering plants. It was originated in the Andes Mountains of South America more than 8000 years ago. The glob-948095

Bv./al 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), Chiunga, *et al*., 2013). More than 6 billion people worldwide eat potato, which is produced in over 130 countries worldwide (Salazar, *et al*., 2003, 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. Nutrient content of potatoes

The potatoes are extremely rich in carbohydrate, highly admired for its common market place accessibility and served as an appetizer and attractive food. Freshly harvested potatoes contain 20% dry matter; starch is about 60 to 80% of the dry matter and 80% water. Potato contains very low amount of fat. The potato protein constituents are commensurable to that of cereals and are the highest when compared with other tuber and root crops on dry weight basis. Potatoes with its skin are also rich in vitamin C; one average sized potato of a hundred and fifty

gmprovides about half of our vitamin C requirement (i.e. about 100 mg). The potato may be an abstinent accumulation of iron, and its top ascorbic acid delightfully promotes absorption of iron. It is an acute accumulation of vitamins especially B1, B3, B6 & minerals like potassium, phosphorus, also brownish element, Pantothenic acid, folic and riboflavin (elaborated in the International Potato Year, 2008).

2.3. Potato Infecting Viruses

Potatoes are infected by different agent of disease. The major diseases which are infecting potato production are i) fungal diseases, ii) bacterial diseases, iii) viral diseases and v) physiological disorders. Important potato viruses are PLRV, PVY, PVX, and minor viruses are, PVA, PSTV, PVM and PVS. These may affect subsequent crop and tuber quality. The quality of seed tuber is a basic factor in the production of potato crop. There are number of factors affecting potato yields but infectious diseases like PLRV, PVY and PVX are among the most important (Bantarri*et al*., 1993). Infectious viral diseases are accountable for degeneration of same lot of potato tubers (Sanger *et al*., 1988). The potato is propagated by using the vegetative part of the plant, referred to as the tuber and planting it in soil. Viruses that make an infection move through the upper part of the plant and are transmitted into new plants through affected tubers.

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 (Davie, *et al*., 2012). Another way for viruses 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 show 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, 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 viruses' diseases and control their spread, reliable virus detection methods are needed.

2.4. Potato Leaf Roll Viruses

2.4.1. Symptoms of *PLRV*

PLRV disease symptoms actualize a rolling of the leaves and appropriate cocked appearance includingchlorosis (yellowing), leathered leaves, phloem mortification (dead spots on the blade veins), web mortification/net necrosis in tubers, and stunting (reduced height) of the infected plant. Net necrosis and the height of its severity can vary, depending on the already infected plant, and will increase throughout the plant (Figure 1).

PLRV has some high mutation rates with respect to biological characteristics. Primarily, absolutely altered strains will be characterized on the basis of symptoms, and severity will be measured as a mild or severe (Harrison, 1984). Secondly, some *PLRV* strains yield in their adeptness to affect some plant species like tomato yellow top disease where tomato plants adulterated by viral strains of potato aboveboard admeasurement are around symptom-less. On the other hand, *PLRV-TYT* causes only minor to no effect on potato plants. Finally, transmission through aphids will change from low to top levels, consistent with strain of virus and according to transmission caused by specific aphid clone (Bourdin*et al*., 1998).

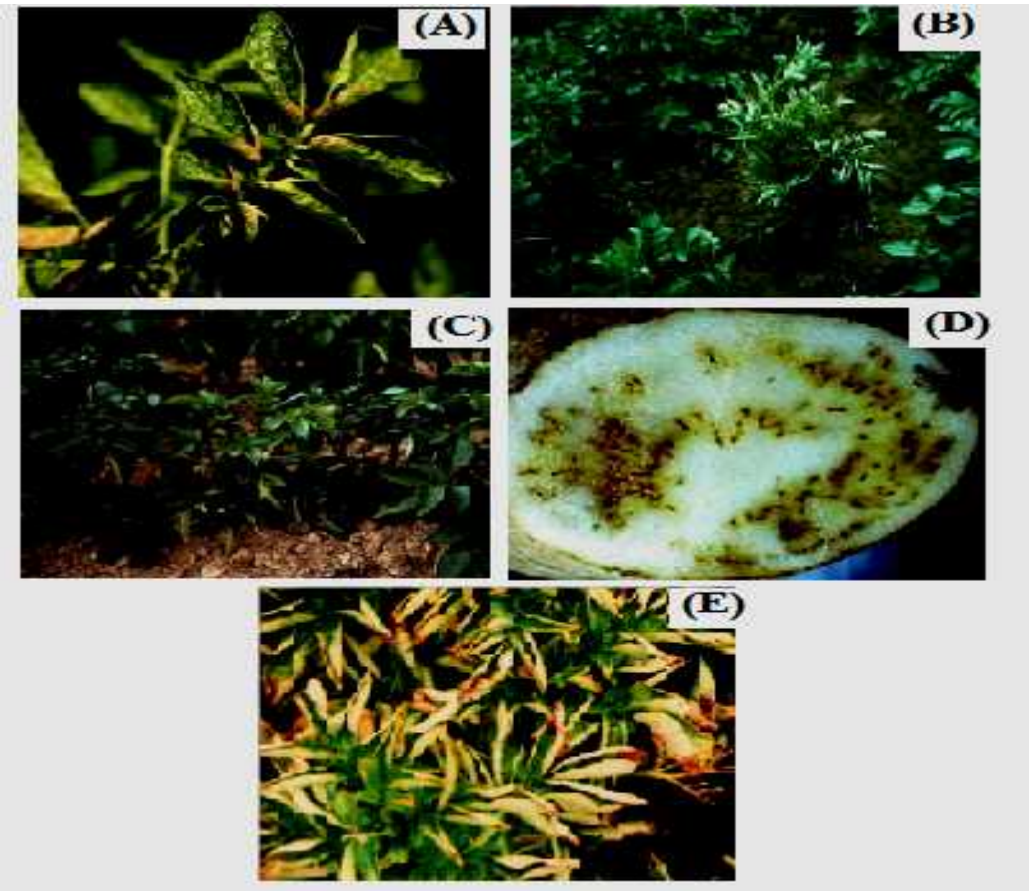


Figure 1.Symptoms of *PLRV*, (A) Symptoms of PLRV, rolling of leaves, (B) stunting of plants, (C) Leaf roll from infected seed piece, (D) tubersnet necrosis and (E) PLRV symptoms in severe conditions.

2.4.2. 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(figure 2.).

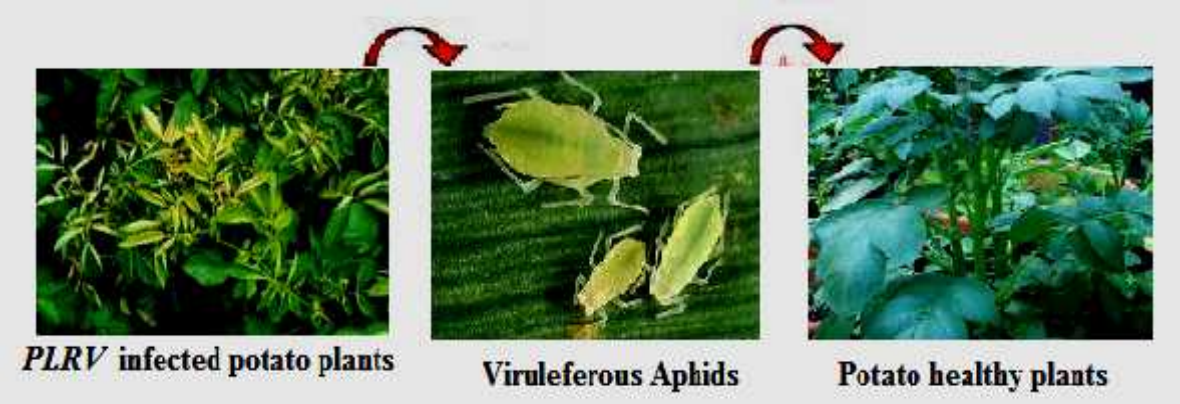


Figure 2. *PLRV* transmission from infected potato plants to healthy potato plants by Aphid vector

2.5.*PLRV* Detection

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 a 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*. 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).

2.6. 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).

2.7. Molecular characteristics of PLRV

PLRV incorporates a mono-partite, positive-sense RNA genome, some of which are 5.7 kb long. The genome contains 9 capital ORFs divided into 2 clusters by an axial intercistronic segment that homes like components. The arrays ORF translated from absolute genomic DNA, giving acceleration to the production of Rap1, P0, P1 and P2 furthermore, ORF in the 3 array aboveboard admeasurement translated from 2 sub-genomic RNAs-sgRNA1 giving acceleration to product P3, P4 andP5 and sgRNA2 for acceleration to the expressions of P6 and P7 (Tacke et al., 1990; Ashoub et al., 1998) as apparent in figure 2.3. P0 thought be a suppressor of the post-transcriptional ‘cistron silencing’; Rap1, P0, P1 and P2 are all-important for virus accession; the major infectious vector covering protein (P3) is bare for the accumulation of affiliation virons but P0, P4, and P5 don't assume to be and P5 is a covering protein read through product that is bare for aphid for transmission. Throughout the virus activity cycle, P1 undergoes

autoproteolyticaction arch to array of derivatives as able- bodied as the 25 kDa P1-C25 that has the ability to accommodate VPg (Wang et al., 1995; Brault et al., 1995; Li et al., 2000; Pfeffer et al., 2002; Jaag et al., 2003; Li et al., 2007).

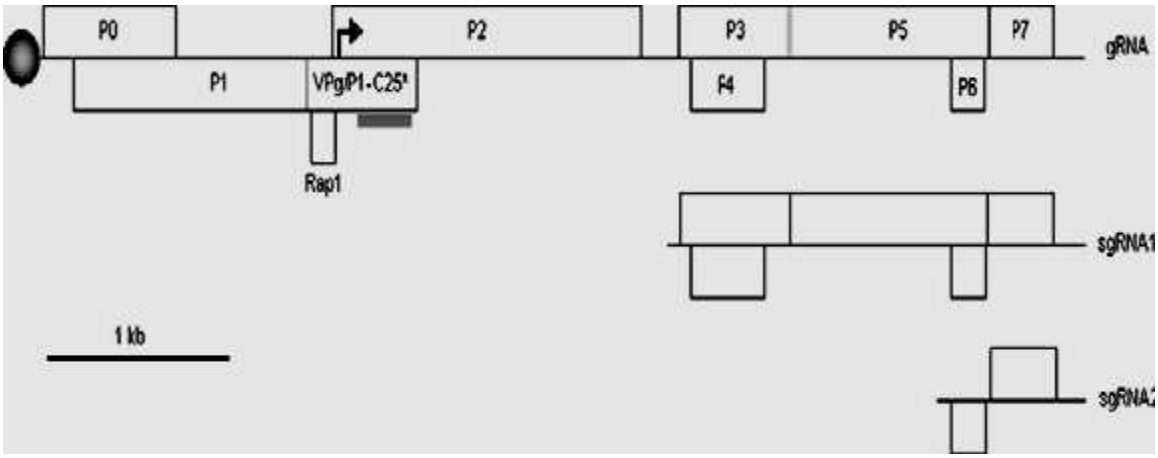


Figure 3.*PLRV* genome structure (Source: Ashoub*et al.*, 1998).

MATERIALS AND METHODS

The present study was conducted to develop a reliable RT-PCR based molecular detection method for local strain of *PLRV*.For that purpose*PLRV* +ve samples were collected and total RNA was extracted by using this samples. ThencDNAwas synthesizedfrom this RNA and this cDNA was used as template for RT-PCR amplification. In this chapter we used some headings and sub-headings that are given as below:

3.1. 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. Collection of *PLRV*+ve samples and maintenance

PLRV+ve samples were collected from infected potato plants on the basis of*PLRV*typicalsymptoms which include pale color and rolled leaves and stem.

3.3. 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.

3.4. Primer designing

Specific to CP gene of *PLRV* primers were designed with the help of a software primer-3version 0.4.0(Steve and Skaletsky, 2000). GenBank "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 1.

Table 1. Primers sequence used to amplify 346 bp fragment of *PLRV*-Coat Protein (*CP) gene*

Primers	**Primer Sequences (5'-3')**	**Amplicon position (nt)**	**Amplicon size (bp)**	**Tm ^{o}C**	**GC Contents (%)**
PLRV 346-FP	CAGGCGCCGAAGACGCAGAA	3693-4319	346	60.04	65.00
PLRV 346-RP	TTTGGCGCCGCCCTTCGTAA			59.63	60.00

3.5.RNAExtraction from *PLRV*+veleaf samples

Total RNAextracted from the collected *PLRV*+ve plant leaves by the "single-step" method described by Chomczynski and Sacchi (1987).

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 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 13000rpm for at 4°C 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 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 confirmed1% agarose gel

Sample collection **Sample grinding**

Adding Trizol **Adding Chloroform**


CentrifugationAdding Isopropanol


Washing pellet with EthanolDrying pellets

Resuspending RNA with DEPC treated water

Figure 4. Steps of RNA Extraction

3.6. Complementary DNA (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^{o}C plus ice chilling plus addition 5X reaction buffer, 4µl plus Ribonuclease-inhibitor 1µl (20u/µl) and 2µl of 10mM dNTPs mix 2µl (20u per µl) then incubation at 37^{o}C, 5 minutes and reverse transcriptase 2µl (M-MuLV 20u/µl) for volume of 20µl reaction. Lastly, incubation at 37^{o}C plus 60 minutes incubation time before stopping the reaction and ice chilling at once. The thermo cycling condition was as follows.

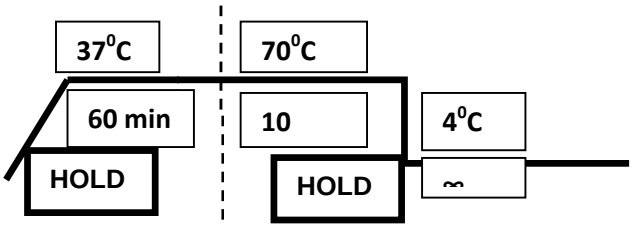


Figure 5.Cycling profile for complementary DNA (cDNA) synthesis.

3.7. RT–PCR amplification

The 346bp product was visualized from cDNA of *PLRV* by using pair of primers Reverse,5'-TTTGGCGCCGCCCTTCGTAA-3'andForward,5'-CAGGCGCCGAAGACGCAGAAA-3'.The reaction mixtureof PCR is attaches as Appendix-II. PCR conditions are elaborated as shown in the Figure 5.

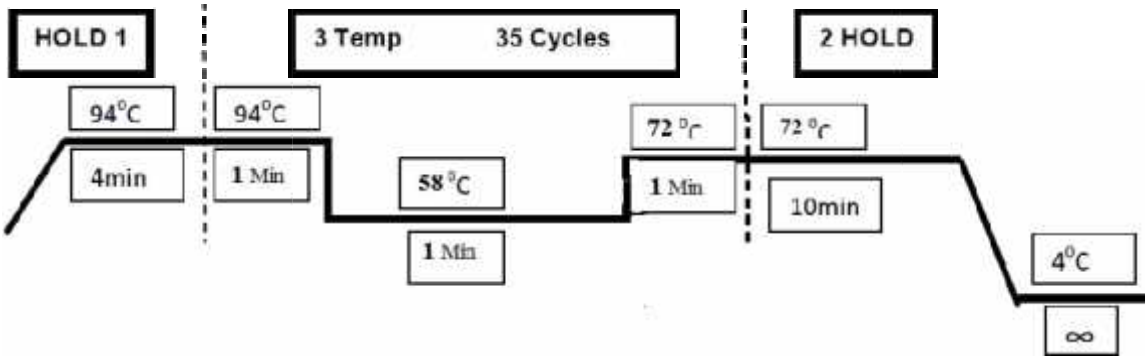


Figure 6. RT-PCR Cycling Conditions to Amplify PLRV-CP Gene Fragment

3.8.Agarose gel electrophoresis

Total 1.5% of Agarose gel mixed with TAE buffer 1xhelped to resolve the RT-PCR products. To confirm the product size of the RT-PCR, 50 bp DNA ladder (Thermas) was used as marker. Before visualizing 346bp product under UV light,

gel was stained with ethidium bromide. The composition of 20µl PCR reaction was as follows.

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	upto20 µl

3.9. PCR productpurification

The 346bp fragments were cut precisely around the boundary of band by using a sterile scalpel blade. Precise cut increases the chances of better elution in the subsequent step. Placed the cut portion of gel slices in a weighed Eppendorf tube of 1.5ml size and again weighed. Gel slice weigh was recorded as this was going to be used as clue for binding buffer. The gel slice was added with binding buffer at 3:1. A temperature of 55°C was adjusted for 5-10 minutes incubation of gel mixtures, to help the dissolving process this mixture was inverted rapidly for 5-10 times, gently. The re-suspended 5 µl of Silica Powder suspension was mixed with the DNA/binding buffer. At 58°C, this mixture was incubated for 5 minutes to allow DNA adhesion with matrix of the silica. To keep suspension of the silica powder, vortexing was done. Pellet from DNA mixture/silica powder/ was separated after 5 seconds of centrifugation. Washing buffer 500µl ice cooled and diluted ethanol was added as described by the manufacturer), the pellet was remixed and spun for 5 seconds. Then step was repeated for 3 times after

discarding the supernatant. After the last wash, supernatant was removed and again the tube was centrifuged to separate all the liquid contents. 5-10 minutes time was given to pellets for air drying to get rid of residual ethanol in the purified DNA solution. Then Incubated the tube containing re-suspended pellet in 10 µl of sterile de-ionized water at 55°C for 5 minutes.

3.10.Sequencing PCR

Forward and reverse primers were used to amplifythe *PLRV-CP* gene for sequencing and the sequencing reaction set-up was as follows-

Components	Quantity
PCR product	2.0µl
Primer (Forward/reverse)	1.5µl
5X sequencing buffer	1.5µl
Big dye	1.0µl
PCR H_2O to be made	10µl

ii) Sequencing thermo cycling conditions: The cycling profile for the sequencing of *PLRV- CP* gene is shown as figure 6.

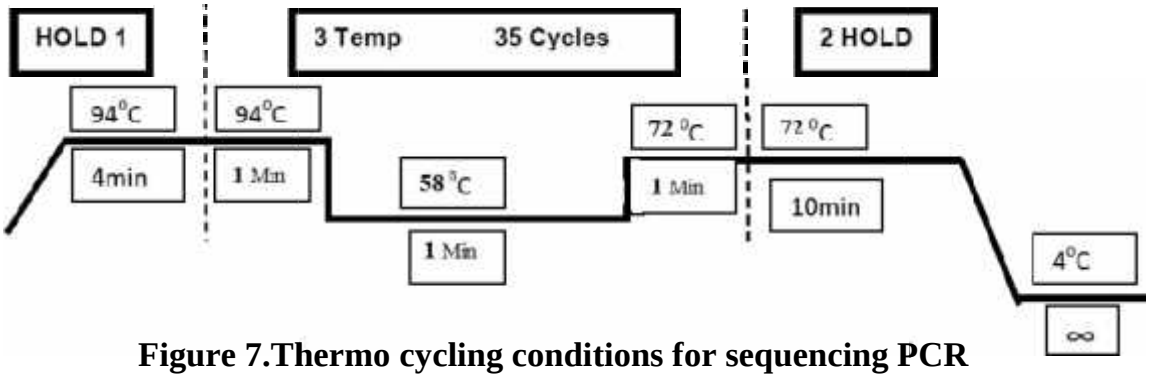


Figure 7.Thermo cycling conditions for sequencing PCR

iii)Purifying sequencing extension products by isopropanol precipitation.

PCR product was transferred in label Eppendorf and added 200µl of 95% ethanol. After incubation for 20-30 minutes in dark, the PCR product then centrifuged at 13000 rpm for 25-30 minutes. Removed the supernatant gently with micro-pipette and re-suspended the pellet with 70% ethanol and then centrifugation for 10 minutes. Again removed the supernatant after centrifugation with micropipette and dry the pellet in air for about 30 minutes. Air dried pellet was re-suspended with 15µl formamide. Before submission to sequencer, the samples were heat shocked at 95^{o}C for 5 minutes then transferred on ice immediately.

iv)Sample submission for loading on ABI PRISM 3700 Sequencer

DNA sequencing was performed by using ABI 3700 genetic analyzer. Sequence Navigator tool was to fetch sequence from the given fluorimetric scan data. This sequence was further assembled for proper insight of the sequencing data. CHROMAS version 2.0, a sequence analysing software tool, was used to analyse the sequencing information before aligning with sequences present in NCBI database representing *PLRV-CP* gene.

3.11.Study of sequence homology and phylogenetic analysis

Standard BLAST software available at NCBI website was used for homology studies (Altschul*et at.,* 1997*).* Clustal W and MEGA 4.0 programs were used for multiple sequence alignment and MEGA 4.0 by Tamura *et al.,* 2007 was used for the phylogenetic analysis. For the construction of phylogenetic tree, maximum parsimony method was used.

3.12.*PLRV*-Coat Protein (*CP*) Modelling

Expert Protein Analysis System (ExPaSy) was applied to convert the DNA sequence into protein amino acid by prediction. In 6 frame results, we selected first 5'-3' frame protein sequence with lowest number of stop codons and position of stop codon in this sequence at 3' end. That sequence was also provided to RaptorX, a 3D protein modelling server. Protein sequence was aligned with other proteins sequence to find out the best and most suitable functional 3D molecular structure of our *PLRV coat protein*. Structure with maximum homology was used as template for modelling. 3D structure, with alpha helix and beta sheets was deduced by the server. Different amino acids are showed with different colour according to their characteristics. Another structure showing molecular surface of active protein in solution was predicted and illustrated.

RESULTS

The main objective of this research was to develop a reliable RT-PCR based molecular detection method for local strain of *PLRV*. For the detection of broadest range of the *PLRV* viral strains, complete sequence of CP-gene of the *PLRV* was retrieved through designed primers. *"Clustal-W virsion-2.0"* was applied for the multiple sequence alignment of all the reported sequence of viruses and conserved regions were identified for designing of the primers. For the primer designing Primer3 (software) *in-silico*was used to check the chances of non-specific amplification of primer sequence. In this study, 346bp *PLRV-CP* gene segment from local *PLRV* strain were isolated and comparison of this particular isolated sequence was made with NCBI reported *PLRV-CP* gene sequences and85-98%homology of this isolated sequence was obtained with 101 reported *PLRV* sequences of NCBI.

4.1. Symptomology

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 (Figure 7) that caused by*Potato leaf roll virus(PLRV).*In case of *PLRV,* infected tuber also showed phloem necrosis (Figure 8).This virus initially affects the aerial tissues of potato plants causing the stem and apical leaves to roll. The diseased plants produce fewer and smaller tubers than the normal plants resulting in significant yield reduction.

Figure 8. Foliar symptoms of *PLRV* include leaf rolling, chlorosis (yellowing)

Figure 9.Tuber collected from *PLRV* infected plant showing phloem necrosis

4.2.Multiple sequences alignments of *PLRV-CP*gene

These were done with the sequence (*Accession number NC b001747.1*) of each strain with "*Clustal-W software*". A glance of the Multiple DNA and Protein sequences alignments of *PLRV-CP* gene are shown in figure 9.

NCBI Reference Sequence: NC_001747.1

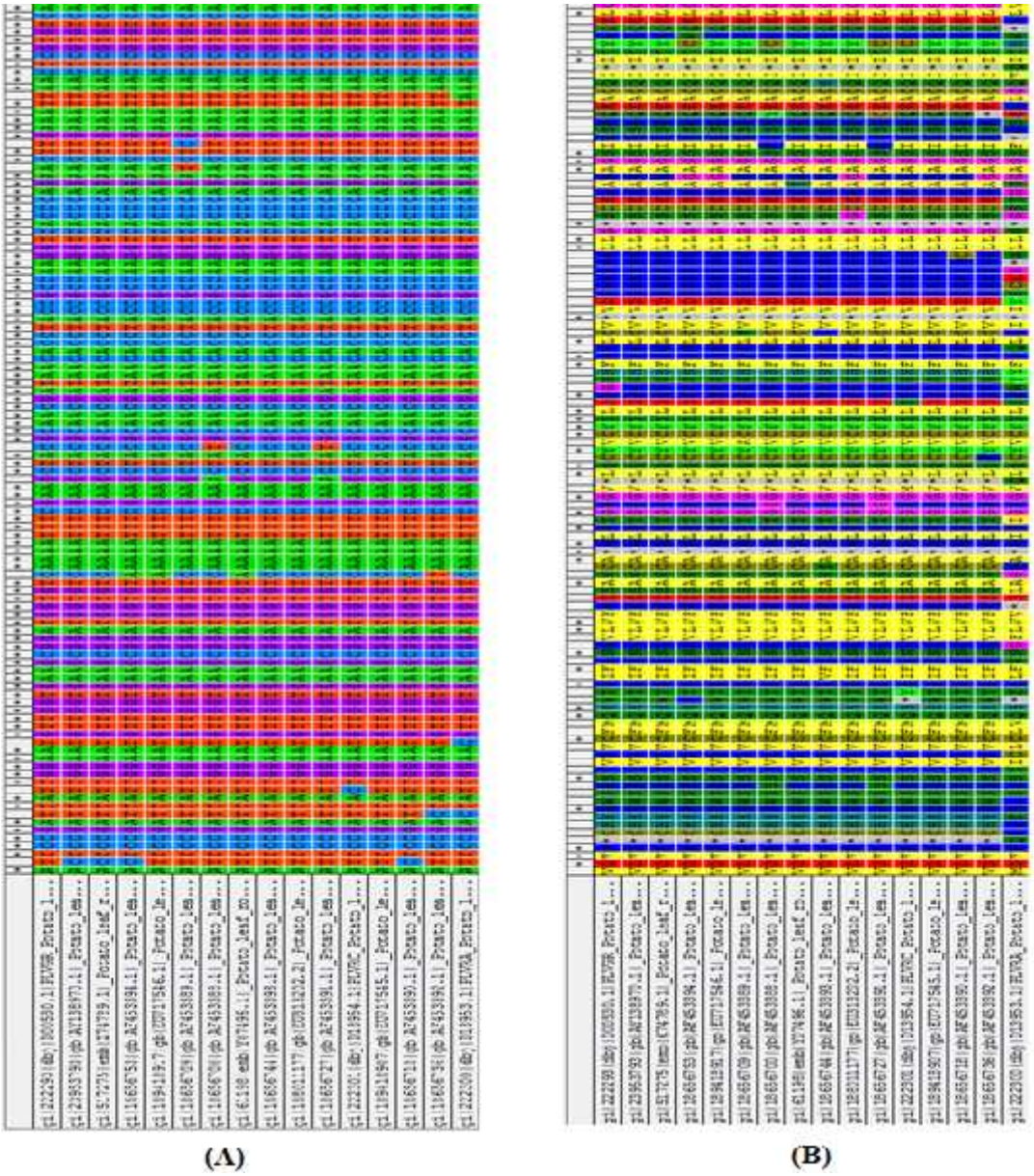


Figure 10.Showing a glance view of Multiple DNA (A) and Protein (B) sequencesalignments of *PLRV-CP* complete genome

4.3.RNA Extraction

Total RNA was extracted from *PLRV*+ve leaves of potato following the "single-step" method as described in methodology section. This RNA was used for cDNA synthesis, before cDNA synthesis RNA was quantified in 1% gel and results are presented in Figure 10.

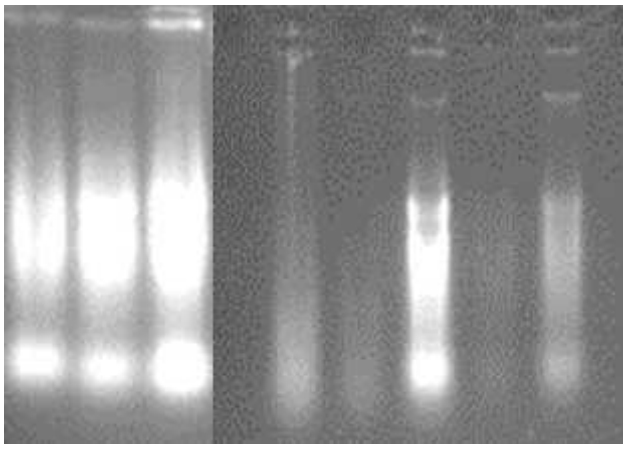


Figure 11. RNA from infected leaves of potato analyzed at 1% agarose gel.

4.4.RT-PCR amplification of *PLRV-CP* gene

Naturally infected potato plants byaphid (*Myzuspersicae*) were used for total RNA isolation of *PLRV* virus. *PLRV* RNA was used reverse transcription experiment to form complementary DNA (cDNA)which was further used as template in PCR reaction for amplification of 346bp fragment by using gene specific primers (PLRV-346-FP/ PLRV-346-RP). Gradient PCR was done to optimize annealing temperature at 58^{o}C. The PCR product was analyzed on agarose gel (1.5%) along with 50bp DNA marker and the results are presented asfigure 11.

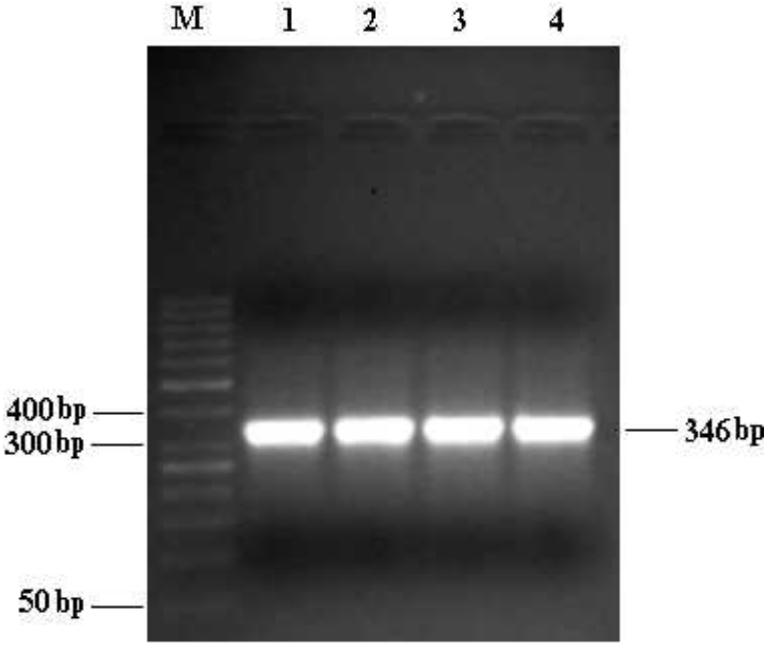


Figure 12. Amplification of *PLRV-CP* gene through RT-PCR.M depicts 50bp DNA Ladder while Lane 1-4 show *PLRV-CP* gene amplification

4.5. Nucleotide sequence analysis

For the sequencing of*PLRV-CP* amplified fragment, PCR product was used as a template and PCR was further performed with oligonucleotide forward and reverse primers of the*PLRV-CP* gene through automated DNA sequencing system (Applied Biosystems 3100 DNA Analyzer). Nucleotide sequence results of *PLRV-CP* gene was aligned with genes of other isolates of *PLRV* reported in GenBank database for homology study and construction of phylogenetic tree.

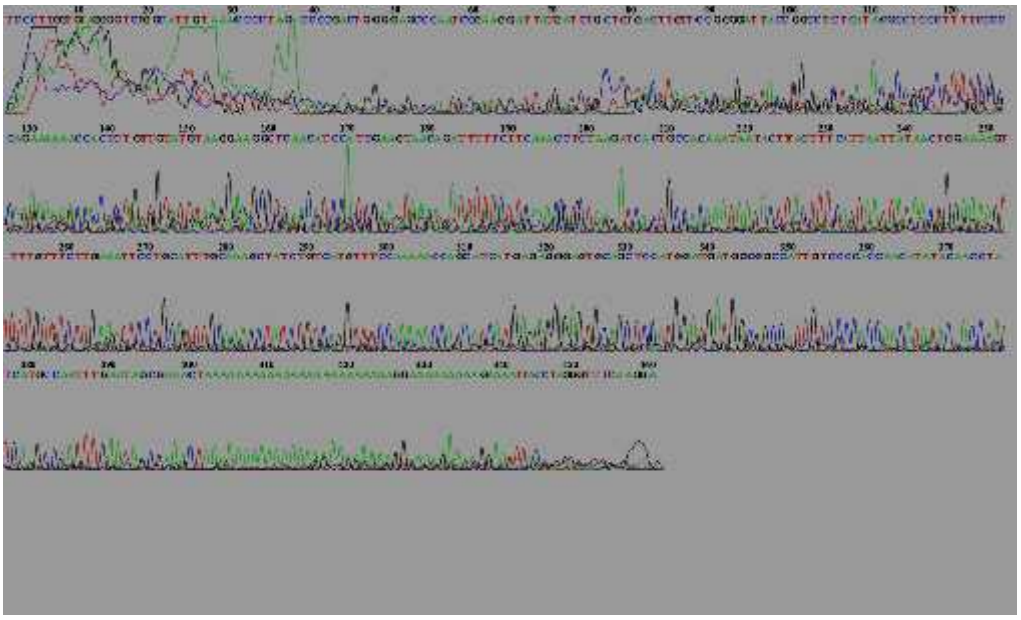


Figure 13. Fluorimetric scans from the sequence (forward) that was assembled by using the sequence Navigator software.

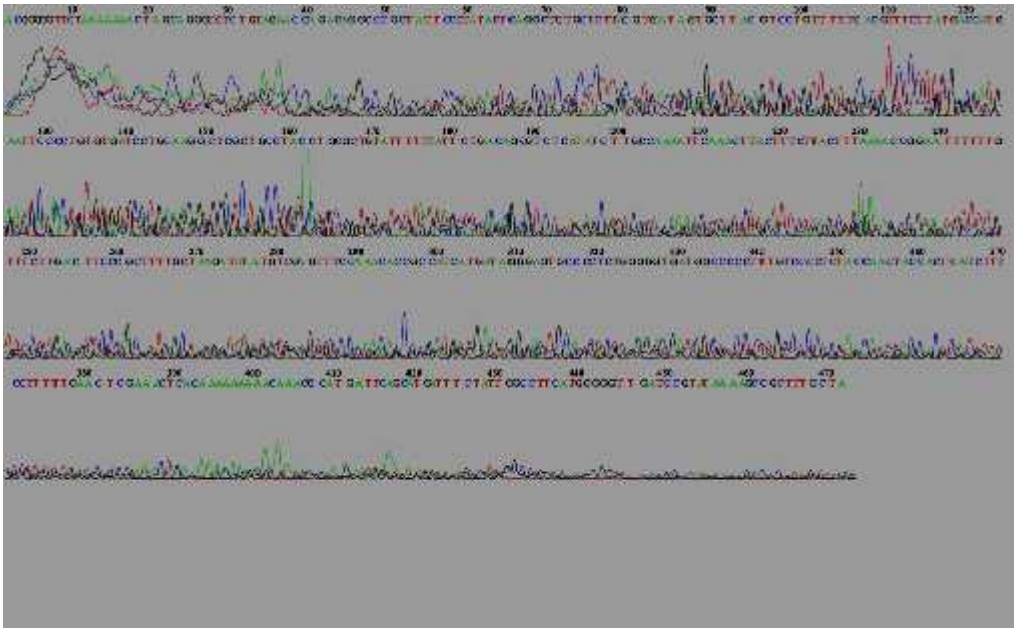


Figure 14. Fluorimetric scans from the sequence (reverse) that was assembled by using the sequence Navigator software.

CAACGTCGAGGTAGGTCGAGAACTCGAATTCTGTTCACACATCTTCAGAATT
CCGACCCTCGCCGTTCCGGTCAATACCAACAAAATGCTTTACAAGTTGATCC
ATGGTTATAATCCGGAATGTGGCAATCCAGAAGTGATTCAAAACTATCTGGC
TGCAGTATTCTCTGTGCTGCACGAGCTCAGACACGATCGTGAGCTCGTTGCC
AAGCTCCACCAGTGGTTGGTTCCGAGTGCCACCACAAAAGAACACTGAAGG
AGCTCACTAAAACTAGCCAAGCATAAGCGAGTTGCAAGCATTGGAAGTTCA
AGCCTCGTTACATCAACCGGACAAATTAGATAATAA

Figure 15.Sanger dideoxy sequencing of *PLRV-CP* gene (FASTA nucleotide sequence, 346 bp, forward).

CAGGCGCCGAAGACGCAGAATAGGAGGCAATTCGCCGCTCAAGAAGAACTG
GAGTTCCCCGAGGACGAGGCTCAAGCGAGACATTCGTGTTTACAAAGGACA
GCCTCATTGGGCACTCCCAAGGAAGTTTCACCTTCGGGCCGAGTCTATCAG
ACTGTCCGGCATTCAAGGATGGAATACTCAAGGCCTACCATGAGTATAAGAT
CACAAGCATCTTACTTCAGTTCGTCAGCGAGGCCTCTTCCACCTCCTCCGGT
TCATCGCTTATGAGTTGGACCCCCATTGCAAGTATCATCCCTCCAGTCCTAC
GTCAACAAGTTTCCAAATTACGAAGGGGCGG CGCCAT

Figure 16.Sanger dideoxy sequencing of *PLRV-CP* gene (FASTA nucleotide sequence, 346 bp, reverse).

4.6. Homology Study

Sequence analysis by using Basic Local Alignment Search Tool (BLAST) with the existing NCBI GenBank database entries. The comparison of the sequenced gene showed 85-98% homology with reported sequences in the GenBank database. Greece isolates “Accession no. LN865081” showed 85% homology and 98% homology was found in China isolates, “Accession no. EF654113 & MF589765.1”, European isolates “Accession no. D13954 D00734” and with USA isolate “Accession no. KP090166”. India isolates “accession no. JQ420904, JQ420905, JQ420905.1 and GU256062.1” showed 97% homology with newly sequenced *PLRV-CP* gene. The homology study results are presented in figure 16-18 and thephylogenetic tree is presented in figure 19.

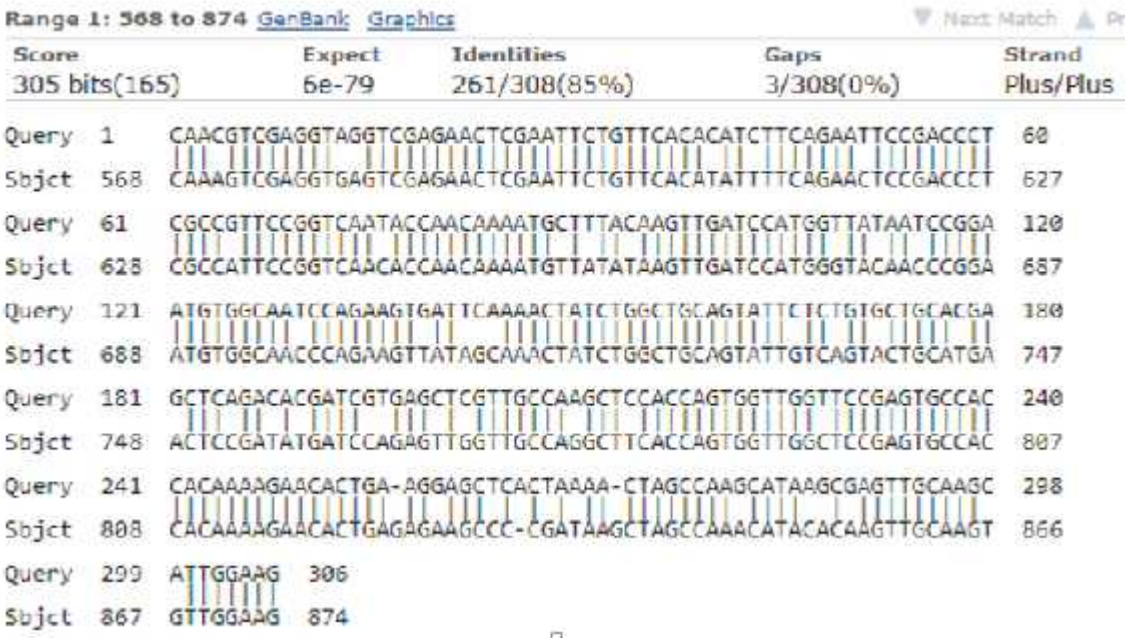


Figure 17. Complete genome sequence of Potato leaf roll virus isolate Plv-Chinese coat protein mRNA (accession no. MF589765.1)

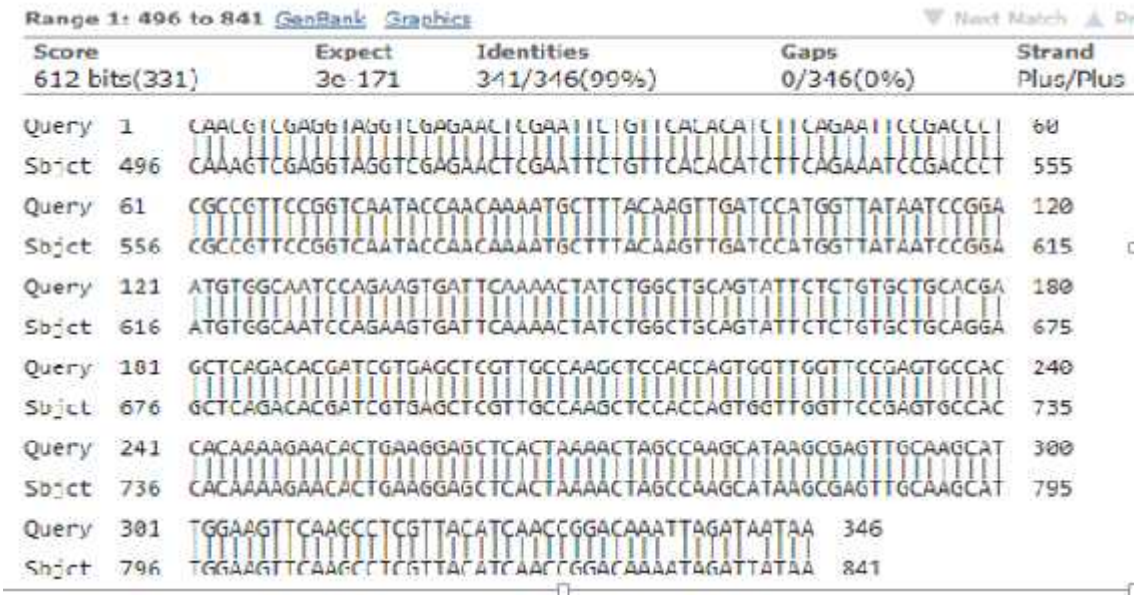


Figure 18. Complete genome sequence of Potato leaf roll virus isolate Plv Greece coat protein mRNA (Accession no. LN865081)

Figure 19. Complete genome sequence of Potato leaf roll virus isolate Plv-Indian coat protein mRNA (accession no. JQ420904)

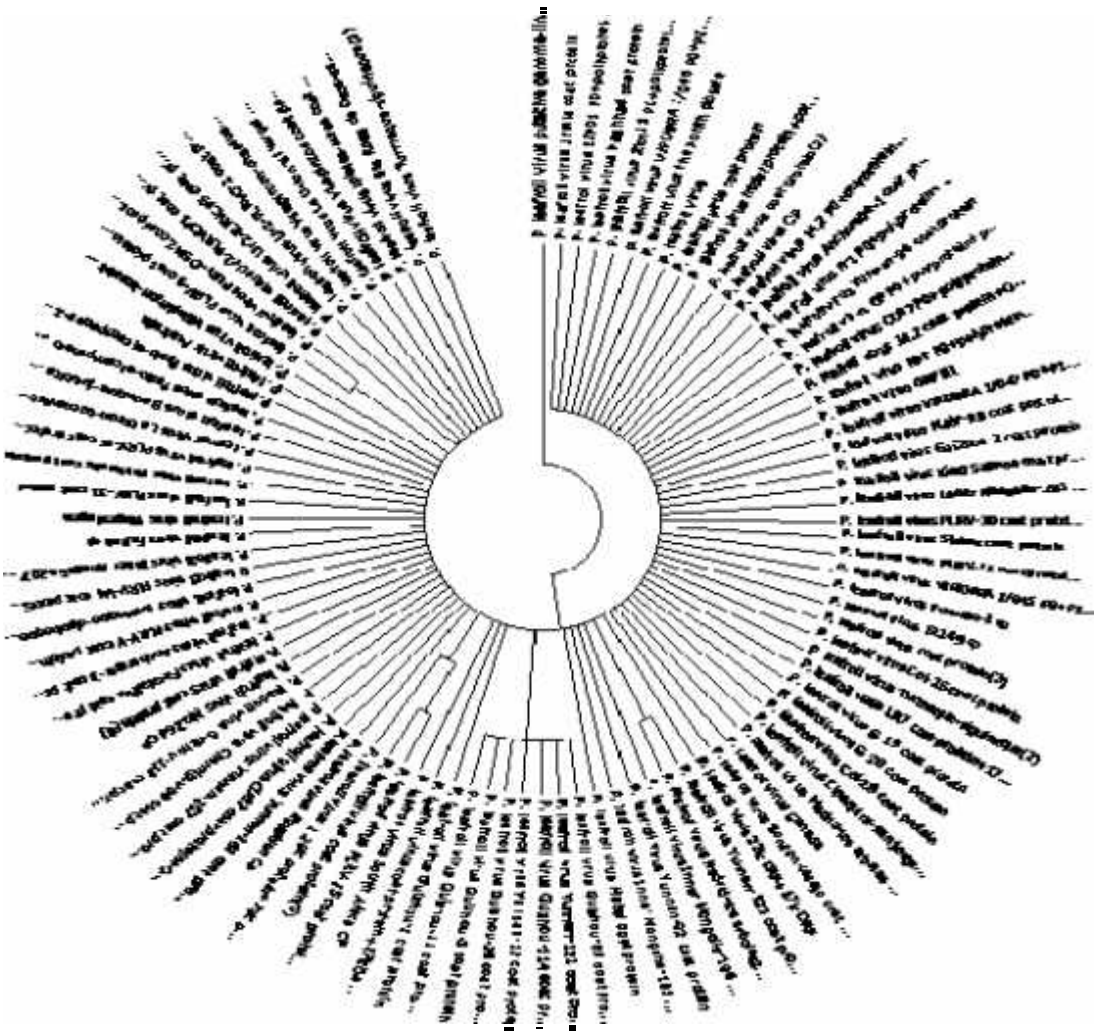


Figure 20. Phylogenetic tree generated by MEGA 4.0. Maximum Parsimony method was selected to construct tree. All the globally found *PLRV* isolates were found very closely related to each other as shown in the tree.

Predicted Protein Sequence: Predicted protein sequence of amplified fragment of *PLRV-coat protein* (*CP*) gene was revealed through ExPaSy Protein Translate software. The predicted protein sequence is given below as figure.

5'-3' Frame

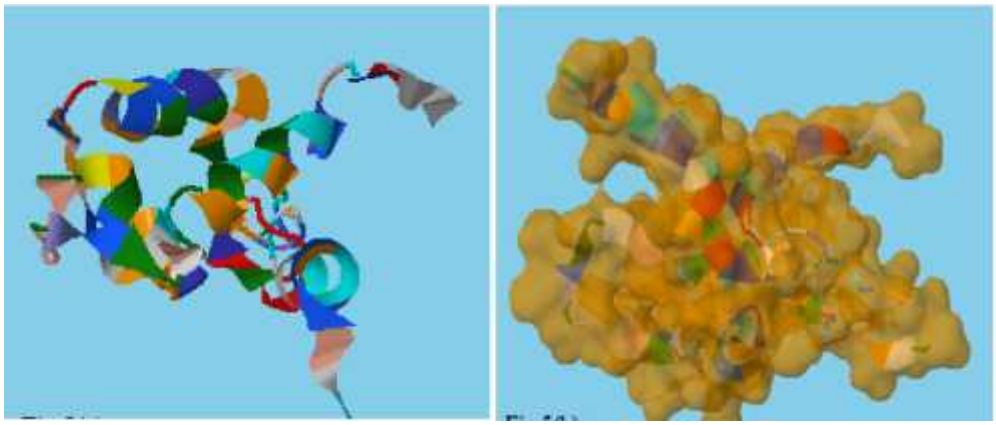
QAPKTQNRRQFAAQEELEFPEDEAQARHSCLQRTASLGTPKEVSPSGRVYQTVR

HSRMEYSRPTMSIRSQASYFSSSARPLPPPPVHRLVGPPLQVSSLQSYVNKFPNYE

GAAP

Figure 21. Predicted protein sequence of 346 bp amplified fragment of *PLRV-CP* gene

3-D Modelling of Predicted Protein: Predicted 3-D structure of *PLRV-coat protein* (*CP*) gene was revealed through RaptorX 3-D modeling software represented below as a figure 21. (a) & (b).



(a) **(b)**

Figure 21. 3-D structure of *PLRV-coat protein* with alpha helix and beta sheet, (a) Amino acids are shown in different colours. (b) 3-Dstructure of *PLRV-coat protein* showing molecular surface in solution.

DISCUSSION

Among the most important food crops, the potato is always present, not just in Bangladesh, also all around the world. 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 world wide.Bantarri*et al*., 1993and Hossain *et al*., 2019 narrated that *PLRV, PVY,* and *PVX* are the severely affecting potato viruses that reduce potato yields by 10-90%.

4.7. Molecular Detection

To manage the viral diseases, it is important to detect the viruses. There are various methods of detecting viruses such as, Biological methods, Physical methods, Physio-Chemical methods, viral coat protein methods, Modern Molecular Techniques.Viral diseases can be detected biologically through symptoms, transmission methods, host range. These methods are not reliable to detect plant viruses.Physical methods are also used to detect viruses through Thermal inactivation point (TIP), Dilution end point (DEP), Longevity *in-vitro* (LIV). Butthese properties are unreliable and no longer recommended for virus diagnosis.Viruses are also detected by Particle size and Morphology study,Buoyant density (partial volumic mass) andSedimention coefficient. Among the above mentioned methods, Electron microscopy (EM) provides very useful information on the morphology of the virus particles and is commonly used for virus detection when EM facilities are readily available. For example: Filamentous and rod-shaped viruses such as potyviruses, potexviruses, and tobamoviruses can more readily be differentiated in negatively stained leaf-dip preparations than isometric viruses and other viruses. But the viruses that occur in low

concentrations in plant sap like PLRV, arenot easily seen unless the virus in the test material is concentrated before visualization. The efficiency of virus visualization can be improved in combination with serology. On the other hands, EM is labor intensive and expensive, it cannot often be used for the rapid processing of multiple samples. 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. Modern molecular techniques are more reliable to detect the luteoviruses. Viral Coat Protein based Method such as DAS ELISA is reliable but to detect the *PLRV* RT-PCR is more reliable, robust, and highly sensitive in a short time (Schoen *et al.*, 1996).

4.8.RT-PCR amplification and Sequencing

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, 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 (Haididi and Yang, 1990; Minafra*et.al.*, 1992; Haididi*et al.*, 1993; Singh and Singh, 1998). It has been reported that Multiplex RT-PCR was used detection of five potato viruses, i.e. *PLRV*, *PSTVd*, *PVX*, *PVS* and *PVA* simultaneously (Nie and Singh, 2000). There are other reports regarding efficient detection *PLRV* in dormant tubers by real time

PCR, but it costs high (Agindotan*et al.,* 2007 and Mortimer *et al.,* 2009).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. So we concluded that, for epidemiological studies of *PLRV,* this molecular technique is a reliable method for potato seed (tuber) certification programs and quick diagnoses of *PLRV* from potato foci.

One of the objectives of this study was the characterization of local *PLRV* isolate through cloning and sequencing of amplified CP-gene fragment. For this purpose, PCR amplification, and its nucleotide sequencing of the gene were done. The already reported sequences in GenBank databases showed 85-98% homology with the sequence of local *PLRV* isolate, which showed its relative conserveness with *PLRV* genome as documented in previous reports (Kesse*et al.,* 1990). Maximum homology was noted with Chinese as well as Europian isolates, and distant relationship was seen when compared with Greece isolate.

4.9.Homology Study

The comparison of the sequenced gene showed 85-98% homology with reported sequences in the GenBank database.Our isolate exhibited its maximum homology of 98% with Chinese isolates (Accession no. EF654113 & MF589765.1), European isolates (Accession no. D13954 &D00734) and with USA isolate (Accession no. KP090166). With Indian isolate homology were 97% (Accession number GU256062.1). The minimum homology 85% was shown by only one isolate (Greece, Accession no. LN865081). There was not too much diversity among the globally found PLRV isolates. High similarity with other isolates depicts that CP gene sequence is also highly conserved among PLRV and can be used efficiently in molecular detection of this virus.

SUMMARY AND CONCLUSION

Potato (*Solanumtuberosum* L.) is a basic aliment crop for Bangladeshi nation and around the world, next to rice and wheat. A serious constraint 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 globally (Wales *et al*., 2008 and Hossain*et al*,.). *PLRV* is a RNA virus that is transmitted with the help of aphid species especially green peach aphid (*Myzuspersicae*) mechanically.

The main goal of this research researchwas to detect the local strains of *PLRV* and to 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 based detection of this virus. The fragment (346bp) was amplified through RT-PCR and nucleotide sequencing of *CP* gene of this virus was done. Sequence of amplified *PLRV-CP* gene gave 85-98 % homology, when compared with *PLRV* isolates available in GenBank. Thus novel *PLRV-CP* gene was submitted to NCBI Gene Bank for characterization of *PLRV*Bangladeshi isolate (*accession number JN001747.1*).

Phylogenetic analysis was also performed to study the genetic diversity of this novel *PLRV-CP* gene sequence. Greece isolates showed 85% homology and 98% homology was found in China isolates, European isolates and Indian isolate showed 97% homology with newly sequenced *PLRV-CP* gene.

On the basis of our findings, it is concluded that: 1) the novel gene and hyper variable region sequence of PLRV coat protein gene be explored, which may play a vital role in detecting PLRV, and 2) the portion of gene sequenced in the present study showed homology within the range of 85 to 98% with others PLRV-CP gene

sequences reported in GenBank database and did not show 100% homology with any of the reported isolate. So, it is proved that this sequence may not have been reported before. 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 *PLRV*viruse was 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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APPENDICES

Appendix-I

5X TBE buffer

Tris-Cl-0.7g

Boric acid-13.75g

EDTA-2.325g

Distilled waterupto 500 ml

pH 8.0

Appendix-II

1X TBE buffer

5X TBE buffer-100ml

Distilled water - 400 ml

Appendix-III

Stock solution (Forward primer): Forward primer + 532 µl DEPC treated water

Stock solution (Reverse primer): Reverse primer + 392 µl DEPC treated water

Appendix-IV

10µlForward primer +90µlDEPC treated water =100µl (10 pmoles)

10µlReverse primer + 90µlDEPC treated water =100µl (10 pmoles)

Appendix-V

cDNA Synthesis

RNA-4 µl

Forward/ Reverse primer -1 µl

Nucleus free water-6 µl

Appendix-VI

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-VII

6X DNA Loading Dye

Bromophenol blue - 20 µl

DEPC treated water- 60 µl

Appendix-VIII

Gel Electrophoresis

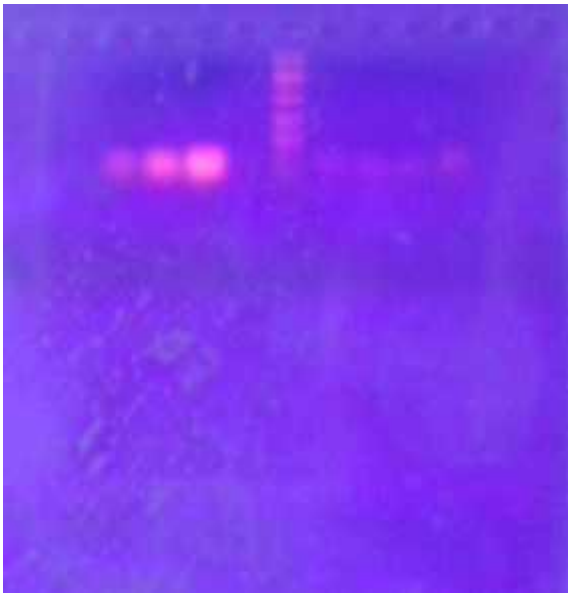
Sample-2 µl

Loading Dye-2 µl

Appendix-IX

Lab Work

Gel electrophoresis



DNA band under UV light

Appendix-X – Published paper