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Serological and molecular detection of Potato Leaf Roll Virus (PLRV)

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Abstract
The purpose of the present study was to confirm the presence of PLRV by DAS-ELISA and RT-PCR. For this the present study undergo serological and molecular detection of potato leaf roll virus (PLRV) by studying PLRV on the basis of symptomlogy, by serological detection (DAS-ELISA) and amplification of coat protein gene by RT-PCR. Ten (10) samples of potato were tested and 8 were found to be infected either by a single virus or more than one virus. Eighty (80)% infection was observed by DAS-ELISA and the resulted cDNA was used in PCR reaction using gene specific primer pair an amplified product of approximately 626 *bp* was observed.

Keywords: *Solanum tuberosum,* Potato leaf roll virus, 626bp, RT-PCR, ELISA

Introduction
Worldwide the foremost food crop is Potato (*Solanum tuberosum*) and is exceedingly prone tonumerous plant viruses counting *Potato virus X*, *Potato virus* and Potato *leaf roll virus* (Family Luteoviridae, genus Polerovirus, PLRV) [1]. The recent studies reported that China claims itself as the chief potato production unit in the whole world [1]. Drastic decrease by 30–50% in yield occurs when infected potatoes produce diseased seeds of transmittable viruses [2]. Potatoes are elevated than wheat and rice in terms of manufacturing and calories per unit area and it gives more yields than cereals economically. Potato is considered as a rich source of carbohydrates, proteins, minerals and vitamins [3]. It mainly exhibits 77.8% water, 2.0 gms protein, 13 gms calcium, 0.06 gms Riboflavin, 25 mg vitamin C, 12 mg ascorbic acid, 0.11 mg thiamin [4]. Potato is known to use as an energy derivative [5]. Irish potato (*Solanum tuberosum* L) was extensively been cultivated long ago and the species is mostly grown throughout the world. In Europe world potatoes are produced and 35% in other states of the world contributes about 25% [6]. The highest annual production of potato in China is 70 million metric tons highest worldwide.

Potato is also an important and most consumable crop of Pakistan. In 2010 Potato creation in Pakistan was rise by rate of 15.9% in contrast to 2009 potato commercial sale [7]. The rate of manufacturing potato crops overall Pakistan is faraway low in difference to other developed states. Due to drop in the manufacturing are attributed different biotic and Abiotic factors. The most important biotic factors include numerous viral diseases caused to potato [8]. Several plant viruses in Pakistan about 83% losses to potato yield [9]. The researchers worked on different diseases prevailing in potato and confirmed that amongst the transmitted viral diseases six are widespread in Pakistan viz. PVA, PVM, PVS, PVX, PVY and PLRV. The incidence rate of PLRV ranged between 15-16% and it is widely distributed in Pakistan [10]. The family Luteoviridae and genus Polerovirus of PLRV as its species [11]. The time span of longevity in-vitro (LIV) at 20 °C is 5 days with optimum Thermal Inactivation Point (TIP) observed temperature ranges from ill Genetic makeup of PLRV possess a single standard RNA along with a coat protein (CP) with a band size of 26.3 Kilo Dalton (KDa) [12]. Made up of 208 amino acids [13]. Ribonucleic acid (RNA) genome composition is of 5,883 nucleotides mainly, PLRV particle size 26 to 30 nm in diameter, with 20 faces or icosahedral symmetry which contains genomic RNA with six large Open Reading Frames (ORF) [14]. It was analyzed presence of ORF in PLRV [15].

ORF protein of the virus gene which possess a size of 28 KDa proteins [16].

PLRV exhibits complex conserved strains which can be notable depends on biological composition and numerous relevant hosts. These PLRV strains are almost similar on antigenic basis [17]. And a virulent PLRV strains helps to protect species *Physalis floridana* L. plants from pathogenic PLRV strains [18]. Hosts are naturally including these species as their suspected hosts *Lycopersicon lycopersicon* (L,) Karst, *Datura stramonium* [19]. Potato nutrient profile illustrated that content of starch and carbohydrates the main energy derivates are quite elevated due to accumulation of these energy derivates in potato leaves exhibiting PLRV infection [20]. High rate of PLRV infection causes net necrosis in potato tubers [21]. The prominent symptoms like leaf rolling and stunting on plants especially because their extent solely depends on the potato cultivar from infected virus tubers [22]. PLRV causes diverse affects eventually yellowing and dryness of leaves tissue in PLRV infection [18]. Transmission of PLRV is by an insect aphid in a non-propagative and circulative way annually and mainly restricted to tissues of phloem by infected plants, which depends upon the time span of reinjection [22]. Although the disease is not transmit by mechanical methods. The only way of transmission is by aphids but it is remained in infected-tuber for years and become long lasting. The virus adversely causes damage onto an infected plant with the rate as 80% but total number of infected plants by the virus field don't out gone from 10% to up and neighbors healthy plants wide along with the non-transgenic plants and speed growing caused to decrease PLRV damages. Damages of the disease have been expressed by average of 15% and danger conditions increase it to 80% [23]. A surveyed was done in Swat District of Khyber Pakhtunkhuwa and about 20 different fields were experimented of potato. PLRV viral infection shown the highest rate of up to 90% and its symptoms were there in most of potato fields with an intense incidence [24]. PLRV is also listed one of most hard task to control over and causes economic losses and unsafe to potato cultivated fields worldwide [25]. The purpose of the present study was to confirm the presence of PLRV by DAS-ELISA and RT-PCR. For this the present study undergo serological and molecular detection of potato leaf roll virus (PLRV) on the basis of symptomology, by serological detection (DAS-ELISA) and amplification of coat protein gene by RT-PCR.

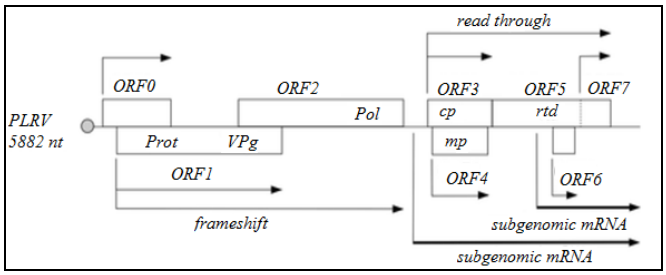

Fig 1: Genome organization of PLRV Abbreviations and genes functions: VPg = Viral genome-linked protein; ORFO = Open Reading Frame 0 act as Suppressor of silencing; ORF1 produced Protein P1;ORF1/ORF2 produced Protein P1-P2; ORF3 produced coat protein, CP Gene; ORF3/ORF5 produced Minor capsid protein; ORF4 produced movement protein.

2. Materials and Methods

The present research work was conducted at the Plant Virology Laboratory, Crop Diseases Research Programme (CDRP), National Agricultural Research Centre (NARC), Islamabad Pakistan.

2.1 Plant Material

Ten leaf samples were randomly collected from the fields at NARC Islamabad, Pakistan.

After collecting the samples from field, they were placed in polyethylene bags, which were properly labeled. On each sample supporting data such as location name, date of collection verity name, symptom and samples number were written and these were kept in an icebox for transportation to plant virology laboratory, these samples were stored at 4 °C in refrigerator until the process of virus detection.

2.2 Elisa Testing

For the detection of viruses i.e. PLRV, PVX, PVY, PVS and PVA in Islamabad city, DAS-ELISA test was used following the method of [26]. All the antibodies and conjugates were obtained from Bioreba and DAS-ELISA was performed as follows**:**

2.2.1 Coating of ELISA plates with IgGs

Each ELISA plate was coated with 1: 1000 dilution of relevant or respective IgGs. In each well of ELISA plates, 100 µl of IgGs diluted with buffer was coated. After coating with IgGs, ELISA plates were incubated at 37 °C for 3 hours. ELISA plates were washed thrice with washing buffer after incubation with an interval of 3 minutes. After washing plates were inverted and dried on blotting paper or towel.

2.2.2 Loading of Sample

To each well of ELISA plates, l00 µl sample extract was added in duplicate, obtained by crushing leaf tissue in extraction buffer, two wells of each plate were used for buffer, healthy and positive control respectively. After loading samples plates were incubated at 4 °C for overnight in refrigerator. After incubation, ELISA plates were washed as described above

2.2.3 Coating of ELISA plates with Conjugated IgGs.

Plated were coated with antibodies conjugated with ALP enzyme (Alkaline Phosphatase).

These were first diluted in conjugate buffer at 1:100, and then l00 µl of this dilution were coated in each well of the plate. After coating plates were incubated at 37 °C for 3 hours. Following the incubation, ELISA plates were washed as described above. These ELISA plates were inverted and dried on blotting paper or towel.

2.2.4 Addition of Substrate

Substrate was prepared by adding substrate tablets (P-nitrophenyl phosphate) into substrate buffer at 0.6mg/ml of substrate buffer. This substrate was added to each well of plates at 150 µl/well. After adding substrate, the plates were incubated in humid place at 35-37 °C for 20-30 minutes**.**

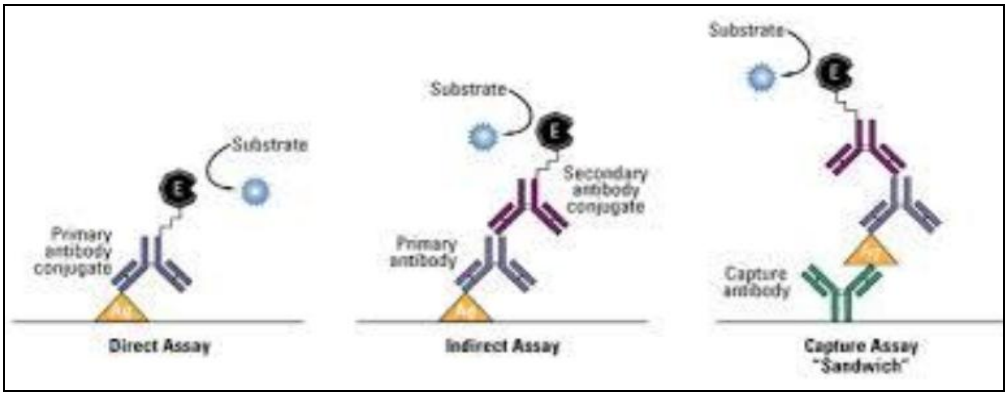

Fig 2: Show the phenomenon of ELISA

2.2.5 Observation

After 20-30 minutes, yellow color was developed in positive wells. Visual observation was made and positive samples were recorded. Now after visual observations we took absorbance reading of each plate at 405 nm in ELISA plate reader. The value, which was equal or greater than twice the mean of healthy and buffer was considered positive.

Fig 3: Loaded ELISA plates for the recognition of six viruses *viz* potato viruses PLRV, PVS, PVY, PVA along with PVM of potato leaf samples and preparation for PCR process.

2.2.6 RNA Extraction

As per extraction method told by [2] total genomic material that is RNA is extracted by potato tubers source. Tubers amount of (300 mg) were stored at freezing temperature at 4 °C in liquid nitrogen and results in fine powder form. To this mixture of 1mL homogenization or mixing buffer was used and the suspension was transferred into a reaction tube β-mercaptoethanol (7µL) 1ml ethanol (75%), and 200µL chloroform, 150µL sodium acetate trihydrate (2 M, pH 4.7) were added up into the mixture in reaction tube then incubated on ice cold temperature for about 15 min before centrifugation or vortex for 25 min (1.01×104 *g*). In mixture present in new tube optimum volume of an isopropyl was added before storage at the temperature 20 °C for 1 hr. Following the same centrifugation or vortex for 25 min (0.73×104 *g*), the pellet formed was in 500 µL disinfected DEPC-treated water or nuclease free water and then phenol as chloroform: was further added and agitate before centrifugation for about 20 min with (1.51×104 *g).* Sodium acetate trihydrate (100 µl) and 1mL isopropyl alcohol were added to the supernatant again as previous method into a new reaction tube and incubated at temperature of 20 °C for 15 min before centrifugation. The supernatant was discarded out and the pellet formed was washed out with ethanol an alcohol and dissolved RNA in it.

Table 1: Detail of ingredients used during the process of PCR.

Concentration	Process	Time
100 mg tissues+ 1ml tri reagent	RT incubate	5min
0.2ml chloroform	Shake vigorously	15sec
RT incubate	10min	
12000g	Spinning	8min
0.5ml isoproponol+ Aqueous phase 60%	storage	10min
12000g	Spinning	8min
Pellet+PPT wall	Supernant draining off	
1ml 75% ethanol	Spinning (7000rpm)	5min
Pellet	Supernant removal	3-5min

Table 2: detailed steps of basic processes of PCR along with ingredients used.

Homogenization	1ml tri Reagent + 50-100mg tissue,5×10×10-6 cells
Phase separation	Homogenate + 0.1ml BCP or 0.2ml chloroform
RNA precipitation	Aqueous phase + 0.5ml isopropanol
RNA wash	1ml 75% ethanol
RNA solubilization	Formazol 0.5% SDS or water.

Table 3: Specific primer sequences designed for PLRV presence.

Primers used	Seq of Primers (5`-3`)	Tm (Melting temp)
PLRV Forward(F)	ATGAGTACGGTCGTGG	50°C
PLRV Reverse(R)	CTATTTGGGGTTTTGCAAAG	56°C

Table 4: RT-PCR formula

Components	Amounts
Nuclease –Free water	Xµl
2 x RT-PCR Quick Master Mix	25µl
0 Mm Mn(OAc)2	2.5µl
Forward Primer	2.0 pmol
Reverse primer	2.0 pmol
RNA Sample	
Total RNA	35.5 µg
Poly (A) +RNA	14.5 ng
Total volume	50 µl

Table 5: RT PCR Program

Process	Temperature and Time
Denaturation	90 °C, 30 sec
RT	60 °C, 30 sec
Pre-denaturation	94 °C, 1 min
Denaturation	94 °C, 30 sec
Annealing	50-70 °C, 30 sec
Extension	72 °C, 1 min
Extension	72 °C, 7 min
Denaturation	90 °C, 30 sec

3. Results

The studies on PLRV were carried out on the basis of symptomology, by serological detection (DAS-ELISA) and amplification of coat protein gene by RT-PCR.

3.1 Symptoms

The potato plants under natural field conditions manifested rolling of lower leaflets. The rolling progressively spread upward and younger leaves turned slightly pale and redness occurred at the tip of rolled leaves. Plants were severely stunted and interveinal chlorosis was commonly observed. Plants were infected with primary leaf roll infection were upright in growth and leaves were rolled and they turned from normal to pale (Fig, 4) A, B, C and D.

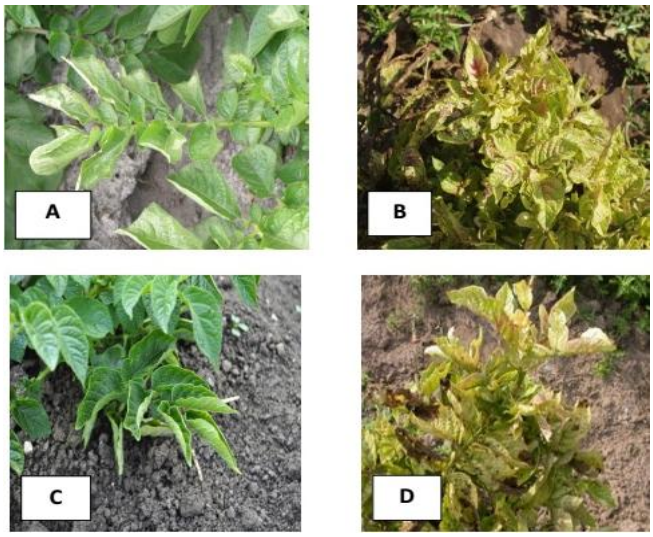

Fig 4: PLRV symptoms observed in potato field (A) and (C) shows leaf rolling, (B) leaf rolling and redness on the tips of leaves and (D) yellowing of leaves.

3.2 Serological Detection

DAS-ELISA was performed for the serological detection of PLRV. All the samples were tested against six viruses namely PVX, PVY, PLRV, PVM, PVS and PVA. The detail results were presented in Table 6.

Table 6: Overall incidence (%) of viruses in samples on basis of ELISA.

Sample No.	PVX	PVY	PLRV	PVA	PVM	PVS	Mixed Infections	Mixed Viruses
Healthy	_	_	_	_	_	_	_	_
1481	_	_	+0.33	_	+ 0.255	_	_	PVM,PLRV
1482	_	_	+0.256	_	+ 0.327	_	_	PVM,PLRV
1483	_	_	+0.280	_	+ 0.279	+ 0.409	2	PVM, PVS,PLRV
1484	_	_	+0.310	_	_	_	_	PLRV
1485	_	_	+0.312	_	+ 0.259	_	_	PVM, PLRV
1486	_	_	+0.290	_	+ 0.311	_	_	PVM,PLRV
1487	_	_	+0.281	_	_	_	_	PLRV
1488	_	+ 3.417	_	_	+ 0.270	_	2	PVY, PVM
1489	_	+ 3.246	+0.260	_	+ 0.266	_	2	PVY, PVM,PLRV
1490	_	+ 3.427	_	_	+ 0.323	+ 0.322	3	PVY,PVM, PVS

3.3 Screening of positive Samples for PLRV Infection

After the confirmation of samples for infection, samples were further screened for Potato leaf roll virus (PLRV) infection as shown in Figure 4.

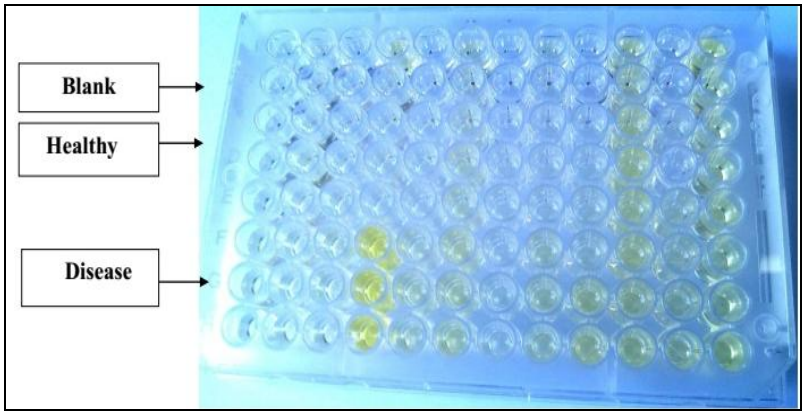

Fig 4: Positive Samples appeared as bright yellow color (W: Microtiter Well)

Percentage of single, double and triple infection was presented in Table 7.

Table 7: Percentage of single, double and multiple infections among 10 samples.

Viruses tested	Single infection	Double infection	Triple infection
PVX	0 (0.00%)	-	-
PVY	3 (30%)	-	-
PLRV	8 (80%)	-	-
PVA	0(0.00%)	-	-
PVM	8 (80%)	-	-
PVS	2 (20%)	-	-
PVS-PVM	-	1 (10%)	-
PVY-PVM	-	2 (20%)	-
PVM-PLRV	-	4 (40%)	-
PVY-PVM-PVS	-	-	1 (10%)
PVM, PVS,PLRV	-	-	2 (20%)

3.4 Over all Incidences of Potato Viruses

10 samples of potato were tested to detect six viruses of potato *viz* PVX, PVY, PLRV, PVM, PVS and PVA. 8 samples were found infected either by a single virus or more than one virus. 80% infection was observed by DAS-ELISA.

3.5 Detection of Single Infection

The samples were found negative to PVX and PVA. Only 4 samples were found infected alone with PVM and PLRV. Whereas 3 samples were found positive to PVY, 8 were positive to PVM along with PLRV and 2 were positive to PVS. The rate of individual virus is 0% of PVX and incidence of PVY is 30%. PLRV incidence is 80%. The incidence observed for PVA is also 0% with no detection of virus in any selected samples. The rate of incidence is 20% of PVS. Rate of PLRV and PVM is high among all the samples and it is 80% because 8 samples were infected with PVM and PLRV.

3.6 Detection of Double and Multiple Infections

In general, 4 samples gave single infections of PVM and PLRV. PVM, PLRV incidence is 40% in four different samples. One sample (1483) found with PVM and PVS, sample (1488) and (1489) had double infection of PVY and PVM. Double infection was found 10% where PVM-PVS combination was present and 20% PVY-PVM combinations were present respectively. Triple infection with PVM, PVS, and PLRV was 20% and PVY-PVS-PVM was 10% respectively**.** The triple infected was observed in sample (1490), (1483) and (1489). In general PVM and PLRV were found prevalent in the samples tested followed by PVY and PVS.

3.7 Graphical representation of Single, Double and Multiple Viruses.

According to the ratio of PLRV infection shown by the graph there was 40% is of single virus, 70% is of double viruses and 30% is of triple viruses rate. The first bar shows rate of single infection and second bar shows rate of dual infection and the third bar shows triple infection rate.

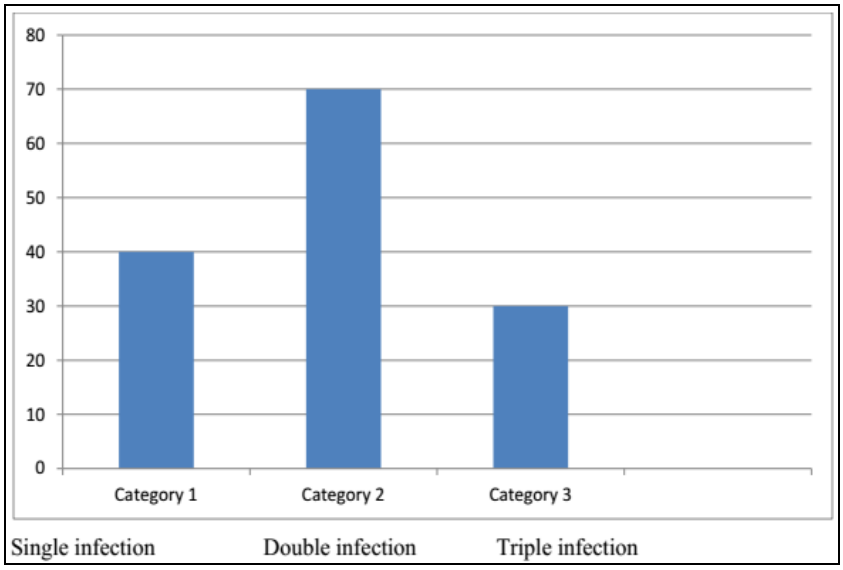

Fig 5: Shows the graphical distribution of single, double and triples infection.

3.8 RNA Isolation

The total extracted RNA was successfully taken from the leaf tissue of potato collected from Green house using tri-Reagent. The extracted RNA was used for cDNA amplification**.**

3.8.1 RT-PCR

The reverse primer of CP gene of PLRV was used for the cDNA synthesis using RNA as
template. The resulted cDNA was further used in PCR reaction using gene specific primer pair. An amplified product of approximately 650 bp was observed (Fig, 5) in UV light after running it on 1% agarose Gel. Similar size of 622 bp CP gene has been reported by [27]. The result of running down the template cDNA is shown in Fig,6 bands were seen via light microscope by placing X-rays film on it the illuminating parts on gel tells us the bands position and helps us in comparing the bands with the ladder or marker whose band size was already known.

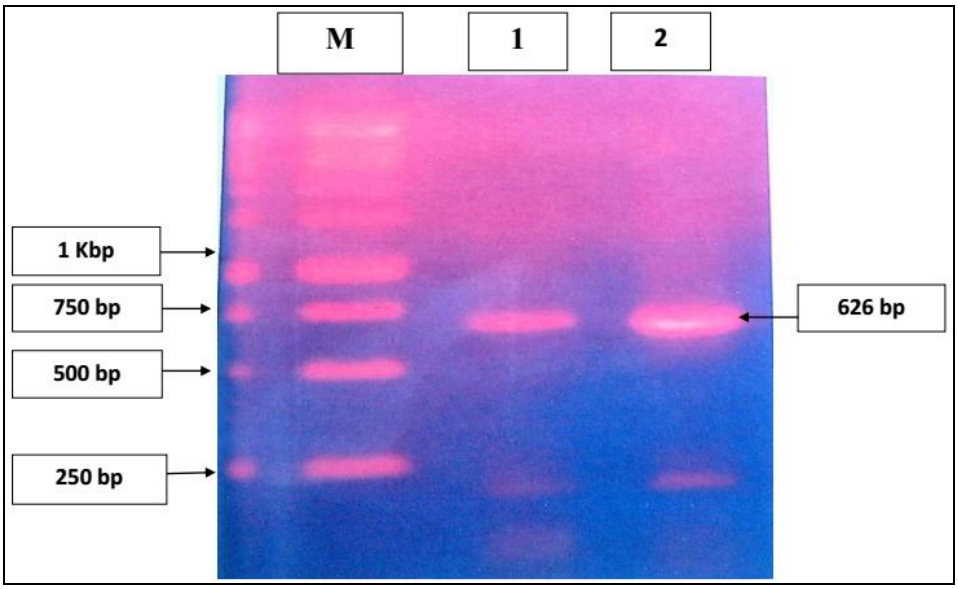

Fig 6: Shows the bands appeared by the process of gel electrophoresis, prevalence of infection shown by band of 626 bp size. Here M represents the known markers and 1 and 2 shows the bands formed of our fragment.

4. Discussion

The six viruses of potato *viz* PVX, PVY, PLRV, PVM, PVS and PVA, 8 samples were found infected either by a single virus or more than one virus. 80% infection was observed by DAS-ELISA.in our study the percentage of PVY is 30%. PLRV incidence is 80%. The incidence observed for PVA is also 0% with no detection of virus in any selected samples. The rate of incidence is 20% of PVS. Rate of PLRV and PVM were high among all the samples and it is 80% because 8 samples were infected with PVM and PLRV. Our study co relate with the previous study [28], These results are comparable to related observations for similar virus strains [29]. These findings are in conformity with earlier work where 15 potato varieties/lines were screened against PLR Vinfection developing from natural inoculum under field conditions [30]. In our study total sample size (1483) found with PVM and PVS, sample (1488) and (1489) had double infection of PVY and PVM. Double infection was found 10% where PVM-PVS combination was present and 20% PVY-PVM combinations were present respectively. Triple infection with PVM, PVS, and PLRV was 20% and PVY-PVS-PVM was 10% respectively**.** The triple infected was observed in sample (1490), (1483) and (1489). In general PVM and PLRV were found prevalent in the samples tested followed by PVY and PVS. In a survey seven main potato growing during autumn season and reported that PLRV, PVX and PVY From 169 fields, 1227 leaf samples were collected and assayed by ELISA. PLRV was detected from 6.54 percent samples, PVX from 13.18 percent and PVY from 23.06 percent samples [31].
The results clearly showed that PLRV infects almost all potato varieties/lines grown in the country. Therefore, extensive screening of potato virus on serological and biological indexing is required to establish a resistance source against PLRV. The system can be applied for preliminary diagnosis of viral load in potato tubers, improve the quality of seed potato and ultimately enhance productivity of potato crop.

5. Conclusion

The viral infection of PLRV in most consumable potato food is a serious problem in Pakistan and worldwide. Current study indicates that the infected potatoes rate fluctuates season to season. Likewise 40% of single virus has the highest rate of PLRV infection, while the double and triple infection rate is 30% and 10% are equally at risk to PVM, PVS, PVY infection. The study also shows that the PLRV levels are more alarming in single and dual infection as eventually 80% of infection was observed by the reliable process DAS-ELISA and the resulted cDNA which was extracted out from the genetic content of under observation leaf samples. This cDNA was used in PCR reaction using gene specific primer pair and in result an amplified product of approximately 626 bp was observed. PLRV may be the dominant virus and it damages leaf rapidly. "Diseased seed harvesting" is one of the major contributing risk factor in single, dual and triple infection.

6. Recommendations

The findings of the current study should be communicated to CDRC crop disease research centers that PLRV screening becomes a regular part of economic destruction day by day. Health care standards/practices also need to be revised since poor health standards contribute significantly towards the spread of these regularly taken diet food infections. Concerned authorities should make general public awareness by conducting seminars, workshops for farmers and provide space for internee in Plant pathology discipline.

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