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Standardization of PCR protocol to check diversity among Pakistani isolates of cucumber mosaic *Cucumovirus* (CMV) infecting chilli pepper

Shomaila Iqbal, Hussain Shah, Muhammad Ashfaq and Ajmal Khan Kassi

Abstract
Research was conducted in the year 2011-12 in NARC to standardize the protocol for the molecular characterization of CMV Pakistani isolates infecting chilli crop. Viral diseases are considered to be the major limiting factors in chili production. *Cucumber mosaic virus* is the type member of the *Cucumovirus* genus having isometric virus particles. It was detected through DAS-ELISA from the samples showing mosaic, leaf distortion, yellowing, and vein etching, stunting and yellowing like symptoms and also confirmed by RT-PCR. For evaluating the diversity different protocols were attempted for the standardization of RT-PCR against CMV. The coat protein (CP) gene of the CMV was amplified using RT-PCR with CP Gene-specific primers. Two steps protocol was finally followed with certain modifications; finally amplification product of an expected size [Australian Capsicum isolate (233 bp), Indian Caulicut Pepper isolate (232bp), Guangdong pepper isolate (218bp) and Indian longum pepper isolate (207 bp)] was obtained. Amplified product showed clear a band of an expected size whereas no satellite RNA was detected by using different sets of primers which shows that Pakistani isolate of CMV infecting chilli pepper belongs to 1b group.

Keywords: *Cucumovirus, Capsicum anum, Cucumber Mosaic Virus,* RT-PCR, ELISA

1. Introduction
Chilli pepper (*Capsicum* spp.), member of family Solanaceae, is both a vegetable and a spice crop of significant economic value in Pakistan [29] and is a rich source of vitamins A, B, C, and G [16, 7, 1]. It is an essential component of daily diet and provides an excellent income generating opportunities to small farmers [31]. Chili production has an economical impact in local as well as export markets in Asia and other parts of the world. More than one billion people consume chili in one or another form on a daily basis and is widely distributed throughout the tropical and subtropical areas of the world particularly India, Pakistan, Indonesia, Philippines, and America [49]. Chilli pepper is more prone to number biotic factors which reduce the quantity and quality of the produce; among them viral diseases are most devastating that causes heavy losses of chili pepper crop [52, 11]. *Chili leaf curl virus* (CLCV), *Cucumber mosaic virus* (CMV) [48, 26] and *Chili vein mottle virus* (ChiVMV) have been reported as most destructive viruses affecting chillies in the world [44, 26].
Cucumber mosaic virus (CMV) belongs to the *Cucumovirus* group of the *bromoviridae* family; is one of the most economical, destructive and occurring worldwide distribution in temperate and tropical areas. The virus was first reported in 1916 [17,18] and since then reported to cause disease in a variety of economically important agricultural and ornamental crops which has a very wide host range including plants form approximately 365 genera and at least 85 families [34]. CMV has a tripartite ssRNA genome coding for one structural and four functional proteins. It has been classified into two subgroups, I and II according to serological relationships [35]. More recently phylogenetic analysis of a number of CMV isolates led to a further subdivision of subgroup I into subgroups IA and IB [40, 41]. The subgroup IB is suggested to contain the 'Asian strains' whereas other members of subgroup I have been kept under subgroup IA. The nucleotide sequence identity between CMV subgroup II and I strains ranges from 69 to 77%, while above 90% within subgroup [33]. CMV is a multi component virus with a single stranded positive sense RNA. RNAs 1 and 2 are associated with viral genome replication while RNA 3 encodes for movement protein and coat protein. Virus disease complex produced various types of disease syndromes like mosaic, leaf distortion,

yellowing, vein etching, stunting and narrowing of leaves [21, 23, 5] are an important factor contributing to low yield and reduced fruit quality [18, 19, 28, 54]. Plant viruses are dependent on vectors for their horizontal transmission and aphids are the most common and important group of plant virus vectors. CMV is efficiently transmitted in a non-persistent manner by more than 75 species of aphids [23] The coat protein (CP) of CMV is a primary determinant of aphid transmission [10]. Weed hosts function as a reservoir for the virus and serve as primary source of inoculum for the development of disease epidemics. Transmission through planting materials is also significant in some crop and weed hosts [25].

In Pakistan, the incidence of CMV has been encountered in major chili growing areas [44]. To commensurate the work done on CMV so far further research on characterization of CMV Pakistani isolates is needed because of its RNA nature. Therefore, characterization of Pakistani isolate of CMV would facilitate to understand the pathogen (virus) in more detail. The present work was conducted to estimate the genetic diversity among Pakistani isolates of *Cucumber mosaic cucumovirus* in chilli pepper using serological and molecular approaches. Five isolates of CMV from different crops in distant geographical locations in India were studied based on host reactions and sequence diversities in CP gene.

2. Material and Methods

2.1 Survey and sample collection

Surveys were conducted in the year 2010-2011 in major chili growing areas of Pakistan. Random and Non-Random samples on the basis of symptoms such as stunted plants with mottled, puckered and malformed leaves bearing small fruits deformed and marked off coloured sunken areas were collected from the field [26, 14]. Each sample was wrapped in a polythene bags, stored in ice box, brought to the plant virology laboratory and positive samples were confirmed through ELISA [15]. Positive collected and tested samples were treated with liquid nitrogen and lyophilized tissues were kept at -80 ºC for future analysis.

2.2 Immuno-Capture RT-PCR

Five ELISA positives isolates from different locations of Pakistan were used for IC-RT-PCR. Primers from known sequence of CMV of other countries were designed and synthesized as described in the Table 1.

Wetze and choi protocols of Immuno-capture RT-PCR was used with certain modifications [38, 12]. Firstly, thin walled PCR tubes were coated with antisera (100µl) diluted (1:500µl) in carbonated buffer (pH 9.6) and incubated at 37 ºC for 3-4 hours followed by washing with PBST buffer. Infected leaves were crushed (1:4w/v) in PBS containing 2%PVP. After a quick spin, 100µl of infected sap was loaded to pre-coated tubes and maintained at room temperature or overnight in refrigerator at 4 ºC. Next day tubes were washed and then used immediately by adding RT-PCR mixture according to the manufacture instructions.

2.2.1 One step RT-PCR

One step RT-PCR was performed by using Choi protocol against RNA virus [7]. 50µl of master mixture was prepared by using virus specific primers (1µl), dNTPs (5µl), Taq Reaction buffer (10µl), Mn $(OAC)_2$ (5µl), RNase inhibitor (2µl), r^{th} DNA polymerases (1µl), and DEPC treated water (25µl) and added to pre coated IC-RT-PCR tube. All the tubes were kept in thermal cycler by providing specifc amplification program as follows; 94 ºC for 5 minutes, 60 ºC for 30 minutes, 94 ºC for 2 minutes, followed by 35 cycles of denaturation at 94 ºC for 30seconds, annealing at 50 ºC for 30seconds, and extension at 60 ºC for 2 minutes, final post extension were carried out at 60 ºC for 7 minutes.

2.2.2 Two steps RT-PCR

Two steps RT-PCR was done by the following two different protocols.

2.2.2.1 Total Nucleic acid extraction and PCR

Raj protocol for RT-PCR was followed by grinding 100mg leaf tissue sample in 10 ml of extraction buffer (50 mM Tris, pH 8.0; 10 mM EDTA; 2% SDS and 1% BME). Spin at 10,000rpm for 10mintues. Collect supernatant then add an equal volume of phenol: chloroform, vortex by inverting the tubes for 5-8 minutes at room temperature nucleic acid was precipitated by adding 2.5 volume of ethanol (containing 1/10 volume 3 M Na acetate). Nucleic acid pellet was obtained after spinning the mixture at 12,000 rpm for 5 min, then washed with 70% ethanol, dried and resuspended in 100 ml RNAase-free sterile water [37, 38]. Quality if RNA was checked on 1% agarose gel.

The cDNA synthesis was carried out using MMLV Reverse transcriptase (10 U) at 42°C for 90 minute and then add 25µl of master mixture; contain reaction buffer (5µl), dNTPs (2.5µl), virus specific primers[Reverse (1µl) forward (1µl)], DNA Taq Polymerases (0.5µl). amplification was done in the thermal cycler by providing a specific program of denaturation at 94 °C for 3 minute followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 52 °C for 1 minute and extension at 72 °C for 1.30 minute and post extension at 72 °C was given for 5 minute. PCR amplification product was then observed on 0.8 % agarose gel as described by sambrook [42].

2.2.2.2 MMLV Transcriptase

Suehir protocol for two steps RT-PCR against RNA viruses was followed [47]. Master mixture for 25µl reaction was prepared by adding Taq Reaction buffer (5µl), Random Hexamer (0.5µl), RNase inhibitor (0.5µl), dNTPs (2.5µl), oligo (dT) (0.5µl), $MgCl_2$ (2.5µl), virus specific primers (1µl), MMLV reverse transcriptase enzyme (0.5µl), DNA Taq Polymerases (0.5µl), and DEPC treated water (10.5µl). The amplification program was followed as 1 hour at 42 ºC for reverse transcription, followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 45 seconds, and extension at 72 ºC for 1 minute followed by final extension at 72 ºC for 10 minutes. The amplified product was run on 1% gel.

2.2.3 Total RNA extractions from CMV infected samples

The total RNA extracted from CMV infected plant material were done by following Spiegel protocol with certain modifications [46]. 100mg of infected plant material were homogenized in ELISA extraction buffer (1:5w/v). 1 volume of cold Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added in 400µl of infected sap. Vortex and centrifuge at 12000xg for 10 minutes at 4 ºC. Supernatant was collected in a new tube and add 1 volume of cold chloroform. Vortex and centrifuge at 12000xg for 10 minutes at 4 ºC. Again collect the supernatant to a new tube and add 1/10 volume of 3M sodium acetate (pH 5.5) and 2.5 volume of absolute ethanol. Store for 2 hr at –20 ºC or overnight at –20 ºC and then follow centrifugation at 12000xg for 20 minutes for the formation of the pellet at bottom. Supernatant was discarded and wash the

pellet with 70 % Ethanol. Air-dry the pellet and resuspend in 100ul of DPC treated water or TE buffer.

2.2.3.1 Quantification
The integrity and quality of total RNA was checked in 1% agarose gel whereas concentration was quantify in spectrophotometer at 260λ/280λ; 5µl of RNA diluted by adding 95µl of DEPC treated water.

2.2.3.2 RT-PCR (Reverse transcriptase PCR)
CMV Primers were designed from known sequences of CMV isolates of different countries already available in NCBI database as shown in Table 1. Total RNA was taken as a template in RT-PCR by using different primer sets. Master mixture of 25µl was prepared both for one step and two step protocols. RT- PCR reaction was carried out in thermal cycler (Primus 25 advanced, PeQLab) by denaturtion at 50 ºC for 30minutes and 90 ºC for 15 minutes, followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 52.5 ºC for 30 second, 53.5 ºC for 30 seconds, 54.5 ºC for 30 seconds, 55.5 ºC for 30 seconds, 56.5 ºC for 30 seconds and extension at 72 ºC for 1 minute final extension at 72 ºC for 10 minute and at 20 ºC for 1 minute.

2.2.3.3 Agarose gel electrophoresis
PCR amplified products were analyzed in 0.8% agarose gel staining with ethidium bromide (10mg/ml). 10µl of amplified DNA product was mixed in 6X loading dye and load in the agarose gel along with 50bp DNA marker. Electrophoresis at 45V until the bromophenol blue had migrated approximately two third of the length of the gel. DNA band were observed in UV Transilluminator and photographed as described by Sambrook [42]

3. Results and discussion
The present study focuses on the characterization of CMV infection in different geographical areas of Pakistan. CMV is most devastating pathogen worldwide with a highest host range. In Pakistan survey, screening and biological characterization for CMV has been conducted in chilli growing areas to estimate the losses by CMV [23, 26]. Through the genetic diversity of CMV Pakistani isolates was done and compare with other isolates of Asia. CMV is one of the most predominant virus diseases of chilli worldwide. The virus exists as a number of strains, but all are apparently capable of infecting chilli and differ only in symptom expression [14]. Infected samples from five different locations showed maximum titer in ELISA test were furthure confirmed by RT-PCR specific primers. Pakistani isolates were placed into five groups viz. Punjab, Sindh, Balouchistan, NWFP and Islamabad isolate. In 1999, an effort has been undertaken by Choi *et al*. to design a set of cucumovirus group specific primer which was successfully used to detect three cucumoviruses [12, 14].

For standardizing the conditions for RT-PCR; Different protocols i.e One step RT-PCR protocol [13], two step RT-PCR protocol [46], total RNA extraction form infected leaf samples [47] and also by using viral RNA purification kit for capturing RNA of CMV *cucumovirus*. By changing the titer concentration 1:2, 1:5, 1:10, 1:20, 1:50 (w/v) in ELISA extraction buffer; No product was amplified by using all above mention procedures. Whereas other researchers who developed the protocol they obtained a very sharp band of amplified product by using different sets of primers. By modifying the number of cycles 25, 35, 40 and 45 cycles during amplification of PCR product. No band was observed in 1% agarose gel under UV Transilluminator. Only smear was observed or in some samples a low molecular band has also been observed it mean RNA or cDNA was denatured or the temperature is not suitable for the primer to attach to binding site for amplification. I remove the smear by changing the annealing temperature again no amplification was observed but in some amplified samples I observed two consecutive band as shown in the Fig.1 were seen in UV light whereas in both modified cases master mixture ratio kept constant.

Finally 2 step protocol was standardized with certain modifications by providing the range in annealing temperature during 35 cycles, finally amplification product of an expected size (233 bp, 232bp, 218bp and 207 bp) were obtained. Specific bands of RT-PCR product were observed at the position corresponding to the expected size of the DNA amplification product. ELISA negative samples shows no amplifications as shown in the Fig. 2 and 3

Complete genome sequences of primers designed and synthesized from coat protein region of CMV of different countries and their comparison with Pakistani isolates were shown in the Table 1 and 2. Two step RT-PCR protocol was helpful in amplifying Punjab and Islamabad isolates of an product of exact size i.e. 233 bp by using primers set of Australian capsicum isolates. Whereas Islamabad isolate was amplified and show expected band of 232 bp and 207bp with primers set of Indian Calicut CMV isolates and Indian Longum pipper CMV isolate respectively. No other isolate were shown amplification with these set of primers. Although every isolate of Pakistan (Punjab, sindh, NWFP, Islamabad and balouchistan) shown an expected bands of 218bp by using the primer sets of Guangdong Pepper Isolate. Similarly, these primers were used previously by Raj and arafati) [38, 2] and found similar type of observations [21]. The sequence variation for a particular region is more prominent from 180-210nt position and these hyper variable sequences could be used for designing [29, 32]. Zhuang also developed a simple and rapid detection method for CMV which showed that all the Chinese isolates used belonged to the subgroup I of CMV [53]. Whereas no amplified product was obtain in control healthy samples. Keeping in view their results, it indicates that all Pakistani isolates belonged to subgroup 1b. It shows the highest homology with the sub group 1b. CMV infecting paprika showed the greatest identity with members of subgroup IB [8].

The RT-PCR based detection of CMV subgroup I and II in field samples of tomato, cucurbits and chilli was developed by designing the primers of flanking IR region [19, 44, 45] also sequenced the complete CP gene of CMV infecting banana Phylogenetic analysis of nucleotide along with amino acid sequence of coat protein gene revealed that CMV infecting banana belong subgroup IB.

Subgroup I strains shows severity in terms of symptom and disease development on tobacco [53] and sequence analysis of these genomic fragments showed highest identities and close relationship with Indian strains of CMV of subgroup IB [39].

Table 1: Source and sequences of primers designed and synthesized from coat protein region of CMV of different countries

S#.	Isolate name	Left / forward Primer	Right / reverse primer	Region	NCBI No.	Product size
1	Banana Isolate of CMV	TGATTCTACCGTGTGGGTGA	CGGCGTACTTTCTCATGTCA	CP 3b	U32859	203bp
2	South Korean Paprika Isolate	ACAAAAGTCCCAGCGAGAGA	GGCGAACCAATCTGTATCGT	2b protein	Ay 827561	191bp
3	Guangdong Pepper Isolate	TGTTCCGCTTTTTACCGTTC	TACCAGCGAACCAGTCTGTG	2b protein	Ay 965891	159bp
4	Guangdong Pepper Isolate	AACCAGTCTGGTCGTAACC	TTGGTGGCTTCAGGGTAATC	3bCP	Ay 965892	218bp
5	Lucknow Longum Isolate	AATCAGTGCCGGTCGTAATC	CCTCAGGGTAACGAGGTGA	3bCP	Dq152254	211bp
6	Indian Piper Longum Isolate	GACCGTGGGTCGTATTATGG	ATGCGGCGTACTGATAAACC	3bCP	Dq285569	232bp
7	Australian Capsicum Isolate	TGATTCTACCGTGTGGGTGA	CGAGTGCATCGTCTTTTGAA	3bCP	Aj 585522	233bp
8	UTR of Australian Capsicum Isolate	CCATCCAGCTTACGGCTAAA	GCACCCGTACCCTGAAACTA	3bCP	Aj 585522	-
9	Indian Belur Pepper Isolate	TGTGGGTGACAGTCCGTAAA	AGCACGGCGTACTTTCTCAT	3bCP	Ay 545924	196bp
10	Indian Caulicut pepper Isolate	TGATTCTACCGTGTGGGTGA	AGCACGGCGTACTTTCTCAT	3bCP	Ay 690621	207bp

Table 2: Comparison of Pakistani chili isolates of CMV with CMV isolates from other countries.

Sr. #	Isolate name	Left / forward Primer	Right / reverse primer	Region	NCBI No.	Product size	P	S	N	I	B
1	Banana isolate of CMV	TGATTCTACCGTGTGGGTGA	CGGCGTACTTTCTCATGTCA	CP 3b	U32859	203bp	-	-	-	-	-
2	South Korean paprika isolate	ACAAAAGTCCCAGCGAGAGA	GGCGAACCAATCTGTATCGT	2b protein	Ay 827561	191bp	-	-	-	-	-
3	Guangdong pepper isolate	TGTTCCGCTTTTTACCGTTC	TACCAGCGAACCAGTCTGTG	2b protein	Ay 965891	159bp	-	-	-	-	-
4	Guangdong pepper isolate	AACCAGTCTGGTCGTAACC	TTGGTGGCTTCAGGGTAATC	3bCP	Ay 965892	218bp	+	+	+	+	+
5	Lucknow longum isolate	AATCAGTGCCGGTCGTAATC	CCTCAGGGTAACGAGGTGA	3bCP	Dq152254	211bp	-	-	-	-	-
6	Indian piper longum isolate	GACCGTGGGTCGTATTATGG	ATGCGGCGTACTGATAAACC	3bCP	Dq285569	232bp	+	-	-	+	-
7	Australian capsicum isolate	TGATTCTACCGTGTGGGTGA	CGAGTGCATCGTCTTTTGAA	3bCP	Aj 585522	233bp	-	-	-	+	-
8	UTR of Australian capsicum isolate	CCATCCAGCTTACGGCTAAA	GCACCCGTACCCTGAAACTA	3bCP	Aj 585522	-	-	-	-	-	-
9	Indian belur pepper isolate	TGTGGGTGACAGTCCGTAAA	AGCACGGCGTACTTTCTCAT	3bCP	Ay 545924	196bp	-	-	-	-	-
10	Indian caulicut pepper isolate	TGATTCTACCGTGTGGGTGA	AGCACGGCGTACTTTCTCAT	3bCP	Ay 690621	207bp	-	-	-	+	-

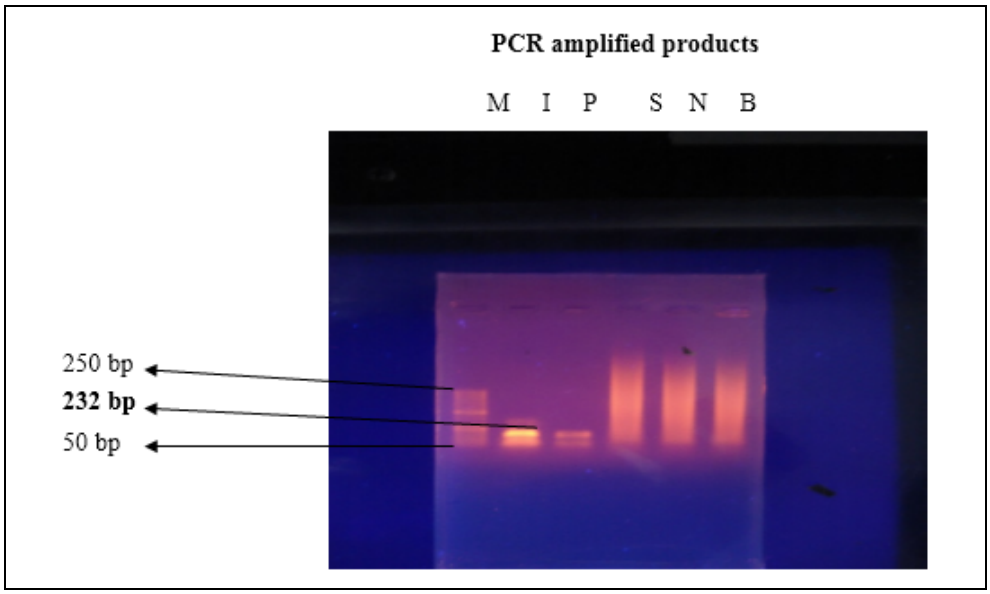

Fig 1: RT-PCR product 232 bp of CMV isolates from Islamabad (I) and Punjab (P) with primer set of Indian Caulicut Pepper isolate (Marker; 50-1031bp)
Abbrivations:P: Punjab, **S:** Sindh, **N:** North West frontier Pakistan, Peshawar **B:** Balochistan, **I :** Islamabad**, M:** Marker(50bp)

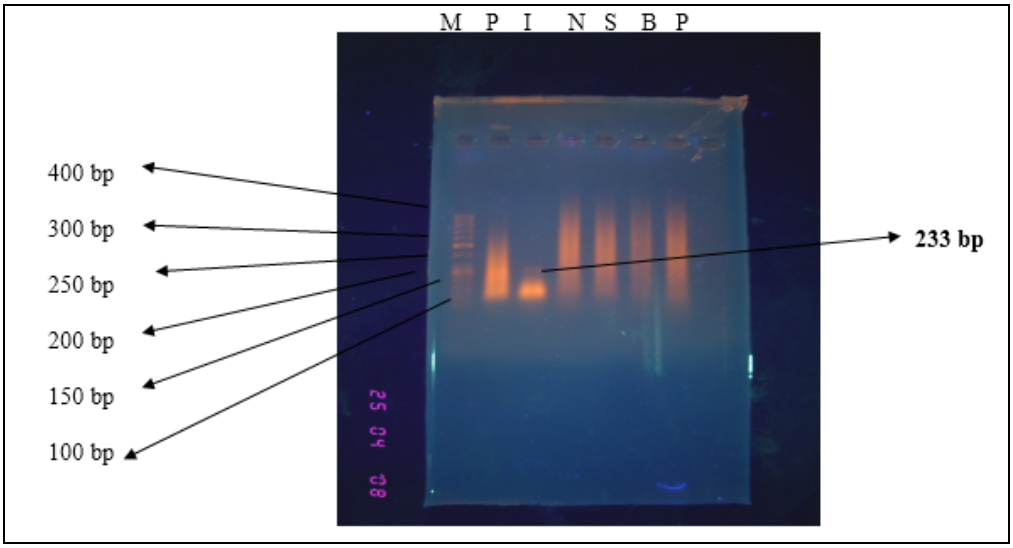

Fig 2: RT-PCR product 233bp of CMV of Islamabad (I) with primer set of Australian Capsicum isolate (Marker; 50-1031bp)

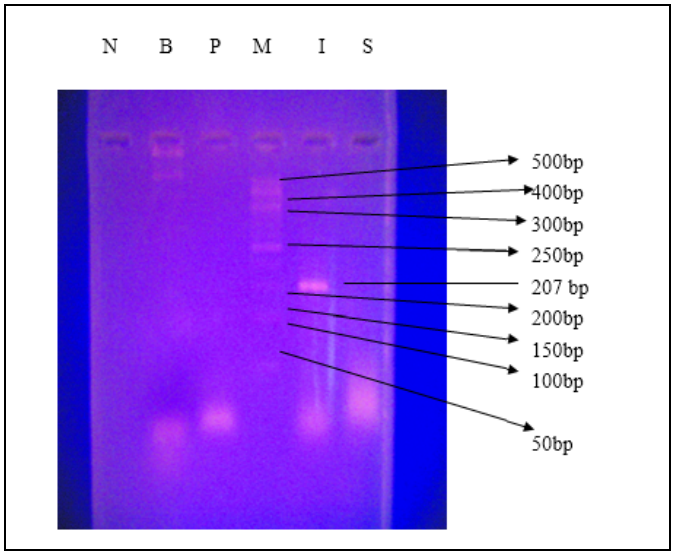

Fig 3: RT-PCR product (207pb) of CMV Islamabad isolate (I) amplified with primer set of Indian longum pepper isolate (Marker 50-1031bp)

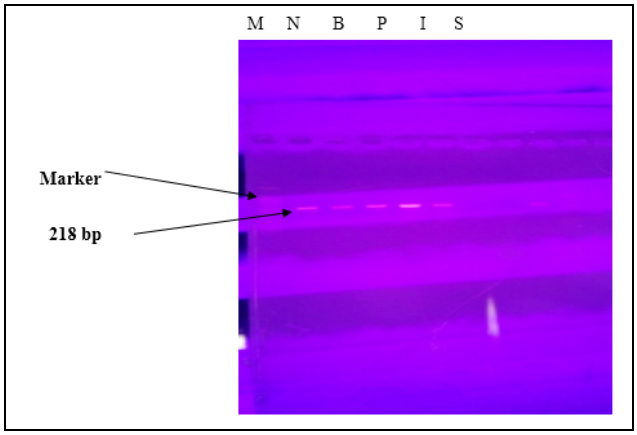

Fig 4: RT-PCR product (218pb) of CMV Islamabad isolate (I), Punjab (P), NWFP (N) Balochistan (B) and Sindh (S) amplified with primer set of Guangdong pepper isolate (Marker 50-1031bp).

4. Conclusion

The genetic diversity studies is useful source for the breeding and genetic researchers to identify resistance gene. Although i have establish and standardize RT-PCR protocol for CMV Pakistani isolates for the researchers to explore more genes to study future its whole genome by focusing on plant resistance to plant viruses including CMV.

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