Pak. J. Agri. Sci., Vol. 57(5), 1353-1361; 2020
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI:10.21162/PAKJAS/20.9773
http://www.pakjas.com.pk

CURRENT STATUS AND GENETIC VARIABILITY OF *CUCUMBER MOSAIC CUCUMOVIRUS* (CMV) ISOLATES INFECTING MAJOR CUCURBITS AND SOLANACEOUS VEGETABLES IN POTHWAR REGION OF PAKISTAN

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Cucumber mosaic cucumovirus (CMV) is a known devastating pathogen and menace threat to several important vegetables worldwide including Pakistan. This study was conducted to determine the incidence, distribution and genetic variability of CMV isolates infecting cucurbits and solanaceous vegetables in the Pothwar region of Pakistan, a rich region for vegetable production. Symptomatic leaf and fruit samples were subjected to double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) using polyclonal antibodies for CMV. Moreover, it was further characterized by Triple Antibody Sandwich ELISA (TAS-ELISA), molecular assays and genetic recombination. The pathogen was prevalent in all districts and detected in all 10 tested vegetables i.e. cucumber, round gourd, watermelon, melon, pumpkin, bitter gourd, ridge gourd, smooth gourd, chilli and tomato, with an average incidence ranging from 11.85% to 25.89%, with the highest incidence in district Attock (25.17%) followed by Rawalpindi (23.04%), Jhelum (18.57%), Chakwal (18.30%) and Islamabad (14.45%). Cucumber, tomato, watermelon and chilli were found to be most affected by CMV with an average disease incidence (D.I.) of 45.38%, 21.15%, 19.39% and 19.05%, respectively. Capsid protein (CP) cistron nucleotides based *In silico* restriction and Phylogenetic analyses revealed that among 10 Pakistani CMV under studied isolates; the four viz., AAJAC, AARpCu, AAHAWM and AACCu isolates were grouped as member of CMV subgroup IB and they shared 94.96% identity with each other while rest of the six isolates viz., AARCF, AARwCu, AARTF, AAHAPu, AAICu and AAJABG grouped in to CMV subgroup II with 92.8% identity among themselves. In recombination detection analysis, the Pakistani CMV isolate AAICu (MH119070) was found likely to be a recombinant between the Indian (X89652) and Pakistani (MH119068) isolates with recombinant breakpoints between 370th and 630th nucleotides. Further research is needed to comprehend the economic impact of the virus and breeding programs for screening of resistant genotypes as per recombinant strains and there is also a need to focus on training of farmer communities to manage disease through adaption of resistant cultivars and controlling vector populations.
Keywords: RNA viruses, CMV, Coat protein, Serotyping, Subgroup I and II, Vegetables.

INTRODUCTION

Cucumber mosaic cucumovirus (CMV), among the most important and devastating pathogens of vegetables, has been reported across the world (Ayo-John and Hughes, 2014). The pathogen causes colossal losses in vegetables especially solanaceous and cucurbit crops (Al-Ali *et al.*, 2013; Akbar *et al.*, 2015). In addition to cultivated vegetable crops, CMV also infects ornamental plants, wild plant species and several weeds belonging to over 100 families (Zitter and Murphy, 2009). The CMV (*Cucumovirus*; family *Bromoviridae*) is a non-enveloped, icosahedral virus with 26-35 nm in diameter that has a tripartite positive sense single-stranded RNA genome containing RNA 1 (3.4 kb), RNA 2 (3.0 kb) and RNA 3 (2.2 kb) segments encapsidated in separate particles. Genomic RNA comprised of five open reading frames (ORFs) which perform multiple functions like host specification, symptoms induction, long-distance movement, inter-viral recombination and virulence determination are controlled by ORF 1, 2a and 2b located on RNA 1 and RNA 2. RNA 3 encrypts two proteins of CMV, capsid protein (CP; translated from subgenomic RNA 4) and movement protein (MP), implicated in viral assembly, cell-to-cell movement and aphid transmission (Shi *et al.*, 2008; Nouri *et al.*, 2014; Ohshima *et al.*, 2016). Serological relations and similarities in nucleotide sequences delineate several strains of CMV into subgroups I and II while phylogenetic analyses divide subgroup I into two groups; IA and IB (Jacquemond, 2012). More than 75 species of aphids transmit CMV in non-persistent manner (Iqbal *et al.*, 2011). Aphids have a wide host range of over 1000 plant species from 101 families including a few monocotyledonous and numerous dicotyledonous plants (Edwardson and Christie, 1991). Important susceptible species include cucurbits, solanaceous crops, cereals, fruits, vegetables and ornamentals (Iqbal *et al.*, 2012).

CMV is one of most important viruses in Pakistan that infects vegetables and causes tremendous quality and quantity losses (Malik *et al.*, 2010; Iqbal *et al.*, 2012; Ashfaq *et al.*, 2014). In Pakistan, more than 45 kinds of vegetables (Ahmad, 2007) are grown over an area of 0.275 Mha with a production of 4.74 Mt (GoP, 2018). Important vegetables grown in the Pothwar region of Pakistan include cucumber (*Cucumis sativus*), round gourd (*Praecitrullus fistulosus*), watermelon (*Citrullus lanatus*), melon (*C. melo*), pumpkin (*Cucurbita pepo*), bitter gourd (*Momordica charantia*), ridge gourd (*Luffa acutangula*), smooth gourd (*L. cylindrical*), chilli (*Capsicum* sp.) and tomato (*Solanum lycopersicum*). Favorable climatic conditions and availability of alternate hosts i.e. weeds during the production season make plantings vulnerable to the insect vectors, which tend to drastically increase during the season and eventually cause major losses. In Pakistan, CMV infection has been reported on a few vegetable species viz., chilli (Iqbal *et al.*, 2012), tomato (Mughal, 1985; Akhtar *et al.*, 2008) and melon (Malik *et al.*, 2010). However, other significant vegetables for CMV infection need to be systematically explored, as most farmers are unaware of the symptoms caused by viral pathogens, resulting in a failure to recognize the diseases and low or poor management measures. Moreover, data regarding the prevalence of the different strains and genetic diversity of CMV infecting major vegetables are quite limited. This study sought to identify and characterize CMV infecting major cucurbits and solanaceous vegetables in the Pothwar region of Pakistan. For pursuing this, intensive surveys were conducted in Pothwar region of Pakistan and collected CMV isolates were serologically and molecularly characterized. Information generated from this research work would be useful for farmers and scientists to develop management strategies against this notorious CMV.

MATERIALS AND METHODS

Field surveys for CMV disease in Pothwar region: CMVD surveys were carried out in the Pothwar region (Attock, Chakwal, Jhelum, Rawalpindi and the capital territory of Islamabad) during 2016-17 (Fig. 1). In each season, 72-75 selected vegetable fields were surveyed and 1120-1156 samples displaying suspected CMVD symptoms such as mosaic, interveinal chlorosis, chlorotic streaks, unusually thick lateral veins with malformed shoestring leaves and misshapen fruit were collected. To facilitate return visits, the surveyed sites were located with Global Positioning System (GPS) coordinates. At least one positive sample after DAS-ELISA attributes field was CMV-positive. A brief questionnaire based on the understanding of the farmers regarding CMV, its vectors and control, as well as data on plant varieties, was administered during the surveys.

Serodiagnosis of CMV: Leaf and fruit samples were subjected to serodiagnosis for CMV in duplicate wells with a commercial kit provided by Agdia Inc. (Elkhart, IN) following the manufacturer's instructions with the modification of Clark-Adams method (Clark and Adams, 1977). Briefly, CMV-specific coating antibody (IgG) was mounted on a microtiter plate by incubating overnight. After washing three times 0.2 mL of the plant sample homogenized in an extraction buffer was dispensed to each well and incubated overnight. Polystyrene plates were coated with alkaline phosphatase conjugated antibodies (Bioreba AG, Switzerland), diluted 1:1000 in conjugate buffer and incubated at 30 ℃ for 5h. In the final step, the microtiter plate was coated with the Substrate Buffer Solution (0.2 mL) comprising the dissolved substratum (pNPP tablet). The reaction was analyzed visually for yellow color development after two hours of incubation and the intensity of the yellow color was measured as optical density (OD_{405nm}) through ELISA reader. At 405 nm, more than the average absorbance value of healthy control samples was considered positive for CMV. Samples showing mild/light yellow color were further tested by reverse transcription polymerase chain reaction (RT-PCR) for authentication whether they were infected by CMV or not. The relative disease incidence was calculated by using the following proportionate test (Rao *et al.*, 2002).

$$\%\ \text{D. I. of CMV} = \frac{\text{No. of ELISA} + \text{ive samples}}{\text{Total no. of tested Samples}} \text{x} 100$$

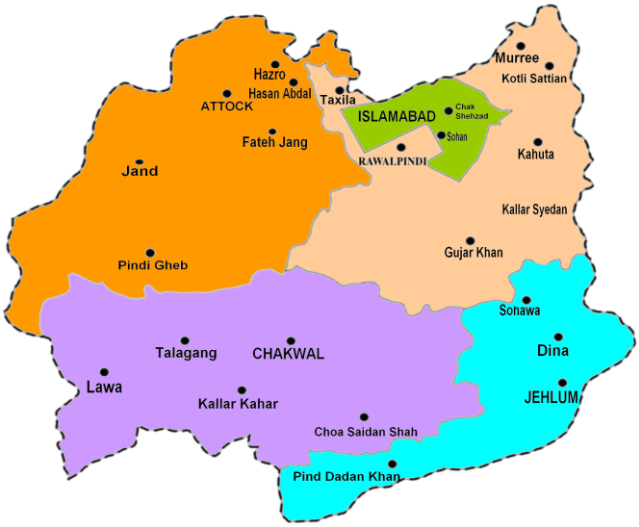

Figure 1. Map of Pothwar region of Pakistan

***Serotyping of CMV isolates using monoclonal antibodies*:** Preserved sap from DAS-ELISA positive samples were used to identify serogroups in Triple Antibody Sandwich TAS-ELISA. For this purpose commercially available TAS-ELISA kits (Cat# PSA 44700/0480 and SRA 44800/0500, Agdia Inc., Elkhart, IN) were used. Positive and negative controls along with poly and monoclonal antibodies were provided by Agdia Inc. TAS-ELISA was performed according to manufacturer instructions with certain modifications as described by Hosseinzadeh *et al.* (2012).

***Molecular detection and sequence analysis*:** Total RNA of the ELISA positive samples along with some healthy vegetable tissues were extracted using the TRIzol ® Reagent (Life Technologies, Carlsbad, USA) enumerated using Nanodrop (Thermo Scientific Co. USA) in line with the manufacturer's instructions. Working RNA dilution @ 500 ng/µL was prepared in nuclease free water and the first strand complementary DNA (cDNA) was synthesized using the RevertAid RT Reverse Transcription Kit (Thermo Fischer Scientific, USA) and CMVR-45 5'-CCC CGG ATC CTG GTC TCC TT -3' (Chen, 2003) as the virus specific reverse primer. The resultant cDNA was used for PCR amplification using the DreamTaq Green PCR Master Mix (2X) (Thermo Fischer Scientific, USA) and coat protein (CP) gene specific primers (CMVF-45 5'-CCC CGG ATC CAC ATC AYA GTT TTR AGR TTC AAT TC-3' and CMVR-45) (Chen, 2003) under the following cycling conditions: initial denaturation at 94 ℃ for 3 min; 30 cycles of 94 ℃ for 30 s, 52 ℃ for 45 s and 72 ℃ for 80 s, followed by a 5-min final extension step at 72 ℃. PCR products were analyzed by 1.0% (w/v) pre-stained agarose gel by electrophoresis and visualized under the U.V transilluminator (Vilber Lourmat, S. No. 6532). The positive amplicons with 1100 bp were purified using GeneJET PCR Purification Kit (Thermoscientific, USA) and cloned into pTZ57R/T vector (InsTAclone™ PCR cloning kit, Fermentas) with chemically competent cells of *E. coli* strain XL1-Blue. Recombinant plasmid DNA was purified using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA) as described by the manufacturer. Digestion with restriction enzymes (EcoR1 and HindIII) validated the existence of an insert in transformants and positive clones were sequenced from Macrogen (Korea) in both directions using M13 forward and reverse primers. The sequences obtained were analyzed using the NCBI BLAST tool and were compared with various CMV strains belonging to subgroups I (A and B) and II. The CMV nucleotide sequences identified in the present study were submitted to GenBank for accession numbers as detailed in Table 3.

***Phylogenetic analysis of the complete CMV CP gene*:** CMV CP gene sequences identified in this study were aligned with respective CMV sequences retrieved from GenBank, belonging to other parts of the world, by Clustal W entrenched in MEGA7 software (Kumar *et al.*, 2016). After aligning their phylogenetic relationship and ancestral lineage was deduced using the Neighbor-Joining method with 1000 bootstrap replicates (to epitomize the taxa's evolutionary history) in MEGA 7 software (Tamura *et al.*, 2013). The nucleotide and amino acid identities of selected sequences were calculated using Sequence Identity Matrix option in BioEdit v7.2.6.1 (Hall, 1999). The nucleotide and amino acid identities were calculated using Sequence Identity Matrix option in BioEdit v7.2.6.1 (Hall, 1999).

***In silico RFLP analysis for strain identification*:** The *In silico* RFLP simulation of the CP gene nucleotide sequences of CMV isolates along with retrieved sequences from GenBank was done using HinfI restriction enzyme in CLC Main Workbench 8.0 (https://www.qiagenbioinformatics.com/). Resulting gel was analyzed for bands differentiation and serotyping of isolates using BioNumerics v7.6 (http://www.applied-maths.com).

***Recombination analysis*:** In order to detect apparent recombinant events in the identified sequences of CMV isolates in Pakistan, aligned sequences consisting of 10 Pakistani CMV and 13 isolates (representing other parts of the world) retrieved from Genbank (Table 3) were analyzed using RDP4 (Martin *et al.*, 2017) by applying the general tab with the default settings which implement all the available methods viz. RDP, BootScan, GENECONV, MaxChi, Chimaera, SiScan and 3SEQ for detection of recombination events among understudied isolates.

RESULTS

***Prevalence and incidence of Cucumber mosaic cucumovirus in Pakistan*:** The study revealed that the CMVD is widely distributed in Pothwar region of Pakistan. The

Table 1. Disease incidence %age of CMV infecting vegetables in Pothwar

Host	2016							2017						
	Attock	Chak-wal	Jhelum	Rawal-pindi	Islam-abad	T. I./T.C	% D. I.	Attock	Chak-wal	Jhelum	Rawal-pindi	Islama-bad	T. I./T.C	% D. I.
Cucumber	22 (40)	10 (25)	8 (21)	32 (68)	4 (13)	76 (167)	**45.51**	22 (41)	10 (22)	8 (25)	30 (69)	6 (11)	76 (168)	**45.24**
Round Gourd	3 (32)	3 (17)	1 (7)	3 (26)	1 (9)	11 (91)	**12.09**	5 (33)	4 (16)	0 (9)	5 (29)	2 (12)	16 (99)	**16.16**
Watermelon	4 (21)	4 (19)	4 (21)	6 (29)	1 (8)	19 (98)	**19.39**	4 (21)	4 (18)	3 (21)	7 (30)	1 (8)	19 (98)	**19.39**
Mellon	4 (21)	2 (11)	1 (5)	3 (18)	0 (7)	10 (62)	**16.13**	5 (22)	2 (12)	2 (8)	6 (21)	0 (6)	15 (69)	**21.74**
Pumpkin	3 (10)	1 (13)	4 (14)	2 (15)	0 (7)	10 (59)	**16.95**	4 (13)	2 (13)	3 (15)	1 (14)	0 (8)	10 (63)	**15.87**
Bitter Gourd	4 (20)	2 (16)	1 (16)	3 (28)	1 (15)	11 (95)	**11.58**	5 (21)	1 (16)	2 (16)	4 (29)	2 (15)	14 (97)	**14.43**
Ridge Gourd	5 (38)	4 (25)	3 (26)	4 (37)	2 (23)	18 (149)	**12.08**	7 (39)	2 (26)	2 (25)	3 (38)	2 (24)	16 (152)	**10.53**
Smooth Gourd	0 (6)	1 (10)	1 (9)	3 (12)	0 (9)	5 (46)	**10.87**	1 (9)	0 (8)	2 (9)	4 (15)	1 (8)	8 (49)	**16.33**
Chilli	8 (40)	6 (33)	5 (31)	9 (52)	4 (23)	32 (179)	**17.88**	9 (41)	5 (32)	6 (32)	11 (51)	5 (22)	36 (178)	**20.22**
Tomato	13 (42)	3 (31)	7 (31)	11 (54)	3 (21)	37 (181)	**20.44**	11 (42)	7 (34)	6 (31)	12 (55)	4 (21)	40 (183)	**21.86**
T. I. (T.C)	66 (270)	36 (202)	35 (181)	76 (339)	16 (135)	229 (1127)	**20.32**	73 (282)	37 (197)	34 (191)	83 (351)	23 (135)	250 (1156)	**21.63**
% D. I.	**24.44**	**17.82**	**19.34**	**22.42**	**11.85**	**20.32**		**25.89**	**18.78**	**17.80**	**23.65**	**17.04**	**21.63**	

disease was detected in all the understudied ten vegetables viz. cucumber, round gourd, watermelon, melon, bitter gourd, ridge gourd, smooth gourd, pumpkin, chilli and tomato crops in all the sampling sites (Fig. 1) that were surveyed. Table 1 shows the percent disease incidence of CMV infecting vegetables in 2016 and 2017.The highest disease incidence of CMV (24.44%) in 2016 was recorded in Attock followed by Rawalpindi 22.42%, Jhelum 19.34%, Chakwal 17.82% and Islamabad 11.85%. While in the year 2017, a relatively higher disease incidence was recorded as compared to the previous year, the highest being in Attock district 25.89%, followed by 23.65% from Rawalpindi, 18.78% from Chakwal, 17.80% from Jhelum and lowest 17.04% from Islamabad (Table 1).

The crop wise study revealed that cucumber had the highest CMV disease incidence in 2016 and 2017 while the lowest was recorded from smooth gourd in the year 2016 and from ridge gourd in the year 2017 (Table 1). Serotyping studies revealed that Chakwal and Jhelum districts had solely CMV subgroup I infection while subgroup II abundantly recorded from Islamabad, Rawalpindi and Attock districts in 2016 and 2017 (Table 2). In the study areas, uneven disease progression was observed during two consecutive growing seasons. Cropping pattern, sowing date, method, temperature, humidity and time of collecting samples were some factors which favored an increase of disease incidence during 2017. Most of the farmers were not familiar with CMVD but they were aware of the presence of aphids in their crops. Although they were aware of aphids, CMVD symptoms were attributed to water deficiency, aphid feeding damage, and/or crop maturity. Many farmers had used insecticides to combat aphid and other insect pests.

***RT-PCR and sequence analysis*:** RT-PCR amplification of total RNAs obtained from ELISA positive samples was performed by the primers CMVF-45/ CMVR-45 (Chen, 2003) specific for the coat protein gene of CMV, and amplicons of 1.1 kb were recorded from infected tissues. No amplification was seen from healthy controls. After careful analysis, 10 selected complete CP gene sequences of CMV from cucumber, pumpkin, bitter gourd, watermelon, chilli and tomato CMV isolates were deposited to GenBank with the assigned accession numbers indicated in Table 3. For each CMV isolate, the sequence contains a complete CP gene of 657 bp along with some portion of 5 and 3′UTR. The BLASTn tool revealed that four out of ten isolates belong to subgroup IB while the remaining six CMV isolates belong to subgroup II. The CMV subgroup IB isolates were 94–95.5% similarity among themselves and 94.2–95.8% to previously reported CMV isolates. When identities were calculated for

Table 2. Serotyping of CMV infecting vegetables in Pothwar

Subgroup	2016							2017						
	Attock	Chak-wal	Jhelum	Rawal-pindi	Islam-abad	T. I./T.C	% D. I.	Attock	Chak-wal	Jhelum	Rawal-pindi	Islam-abad	T. I./T.C	% D. I.
I	30 (66)	36 (36)	35 (35)	27 (76)	2 (16)	130 (229)	**56.77**	29 (73)	37 (37)	34 (34)	24 (83)	2 (23)	126 (250)	**50.40**
II	36 (66)	0 (36)	0 (35)	49 (76)	14 (16)	99 (229)	**43.23**	44 (73)	0 (37)	0 (34)	59 (83)	21 (23)	140 (250)	**56.00**

Table 3. List of CMV sequences used for in silico restriction and recombination detection analysis

Country	Strain	Sub-group	Accession No	Reference
Japan	M2	II	AB006813	Takanami *et al.* (1999)
USA	LS	II	AF127976	Roossinck *et al.* (1999)
UK	Kin	II	Z12818	Boccard and Baulcombe (1993)
Hungary	Trk7	II	L15336	Salánki *et al.* (1994)
Australia	Q	II	M21464	Davies and Symons (1988)
Pakistan	AARP	II	MF100856	Ahsan and Ashfaq (2018)
Pakistan	AARCF	II	MH119066	This Study
Pakistan	AARwCu	II	MH119067	This Study
Pakistan	AARTF	II	MH119068	This Study
Pakistan	AAHAPu	II	MH119069	This Study
Pakistan	AAICu	II	MH119070	This Study
Pakistan	AAJABG	II	MH119072	This Study
Egypt	HM3	IB	KX014666	Unpublished
India	PhyM	IB	X89652	Haq *et al.* (1996)
Pakistan	AAJAC	IB	MH119061	This Study
Pakistan	AARpCu	IB	MH119062	This Study
Pakistan	AAHAWM	IB	MH119064	This Study
Pakistan	AACCu	IB	MH119065	This Study
USA	M	IA	D10539	Owen *et al.* (1990)
USA	Sny	IA	U66094	Gal-On *et al.* (1996)
USA	Fny	IA	D10538	Owen *et al.* (1990)
Japan	Leg	IA	D16405	Karasawa *et al.* (1997)
Japan	CS	IA	D28489	(Chaumpluk *et al.*, 1996)

subgroup II isolates, they were found to be 89.6-92.8% identical with each other while 91-92.3% with other isolates reported from elsewhere in the world. At nucleotide and amino acid levels, more deviation was observed in isolate AAJAC (MH119061) as compared to other Pakistani isolates. In relation to isolates from other countries, the Hungarian isolate L15336 and Egyptian isolate KX014666 shared the least identity (91.6% and 93.4%) with the Pakistani CMV subgroup II and subgroup IB isolates, respectively. The USA LS isolate (AF127976) and Indian PhyM (X89652) were most closely related to the Pakistani CMV subgroup II and IB isolates, respectively.

***In silico restriction enzyme and phylogenetic analysis*:** The HinfI restriction pattern of the CMV CP gene outlined 23 isolates into 3 subgroups: i.e. Subgroup IA, IB and II. Six isolates from this study were grouped in Subgroup II, while four with Subgroup IB isolates. HinfI divided each isolate into 5-8 fragments. Analysis of virtual agarose gel in BioNumerics v 7.06 revealed that only subgroup II isolates contain a distinct 178 bp band while subgroup I isolates had a88 bp band. 127 bp band discriminate subgroup IA isolates from IB. Phylogenetic analysis revealed that all the isolates were assembled into three well-separated clusters in accordance with the sequences of their close relatives in the same subgroups (IA, IB and II) (Fig. 2).

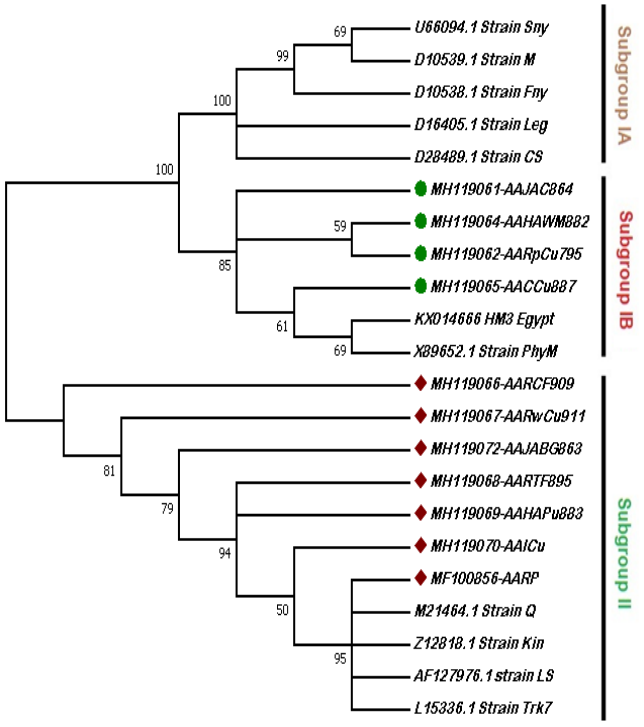

Figure 2. Evolutionary relationships of Pakistani CMV Nucleotide sequences of CP gene with previously reported GenBank sequences.

The new CMV isolates from Pakistan's Pothwar region formed clusters with a number of established standard isolates used as reference isolates for each subgroup such as isolates LS, Q, Kin, Trk7 and M2 for subgroup II; PhyM and HM3 for subgroup IB; Fny, Sny, CS, M and Leg for subgroup IA; all of which are consistent with previous studies (Boccard and Baulcombe, 1993; Chaumpluk *et al.*, 1996; Gal-On *et al.*, 1996; Haq *et al.*, 1996; Roossinck *et al.*, 1999; Takanami *et al.*, 1999). The discrepancy of subgroups I and II is evident in Fig. 2. Four isolates (MH119061, MH119062, MH119064 and MH119065) out of the total 10 Pakistan CMV isolates clustered with the representative isolates of IB group: i.e. PhyM (India; X89652) and HM3 (Egypt; KX014666) while the remaining six isolates of this study clustered with the subgroup II isolates. Our Pakistani isolates of CMV (MH119066- MH119070 and MH119072) form a separate parent cluster with the representative LS (USA; AF127976), M2 (Japan; AB006813), Kin (UK; Z12818), Trk7 (Hungary; L15336) and Q (Australia; M21464). No isolate was clustered with the Subgroup IA isolates. Phylogeny results indicate that in the Pothwar region of Pakistan subgroup II prevailed more than IB while the IA subgroup may not be available.

***Recombination events*:** Recombination detection analysis revealed the recombinant probability of our isolate AAICu (MH119070) with Indian isolate X89652 and Pakistani isolate AARTF (MH119068) in four positive statistical methods implemented within the RDP4 program with a recombinant breakpoint between nucleotides the 370 and 630 (Fig. 3). P values and recombination breakpoints for each recombination event are given in Table 4.

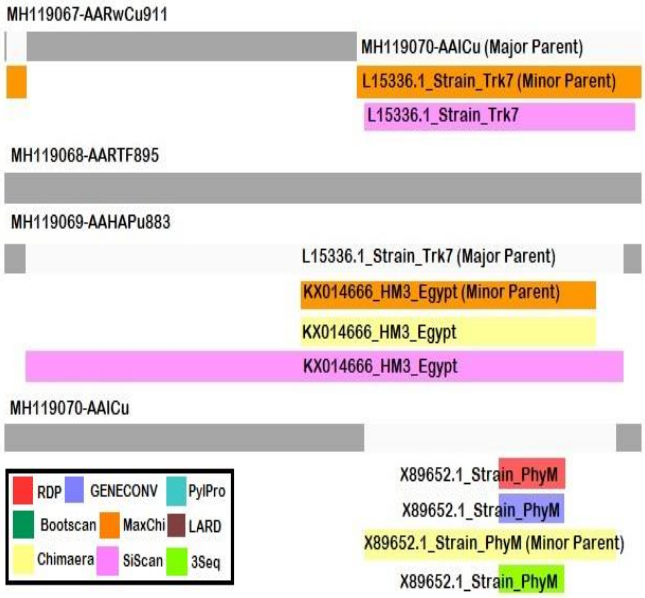

Figure 3. All evidences of Recombination with major and minor parents detected in Pakistani isolates of CMV using all the methods available in RDP4.

Table 4. Recombination events detected in Pakistan isolates of CMV sequences

Recombinant Isolate	Breaking point		Major Parent	Minor Parent	P-Value						
	Starting	Ending			RDP	GENECONV	BootScan	MaxChi	Chimaera	SiScan	3SEQ
MH119067	24	630	MH119070	L15336 strainTrk7	NS	NS	NS	3.512×10^{-2}	NS	2.61×10^{-6}	NS
MH119069	21	637	L15336 strainTrk7	KX014666 HM3	NS	NS	NS	5.719×10^{-6}	5.207×10^{-3}	3.90×10^{-18}	NS
MH119070	370	630	MH119068	X89652 strain PhyM	6.339×10^{-5}	4.023×10^{-3}	NS	NS	2.435×10^{-2}	NS	4.876×10^{-3}

DISCUSSION

This study demonstrated the pervasive occurrence of CMV in cucurbits and solanaceous crops in the Pothwar region of Pakistan. It implies that this disease has been existing in the field since it has been identified for a long time and confirms the results of previous studies (Mughal, 1985; Akhtar *et al.*, 2008; Malik *et al.*, 2010; Iqbal *et al.*, 2011, 2012; Ashfaq *et al.*, 2014; Ahsan and Ashfaq, 2018). The prevalence of CMV from pumpkin and bitter gourd are here reported from Pakistan for first time. Although data regarding yield losses were unavailable, the higher disease incidence in this region was a cause of concern as premature crop defoliation was attributed to the disease, which ultimately had an adverse effect on fruit size, yield and quality of crop. The occurrence of lesions in the leaves had a negative impact on the marketability and edibility of leafy vegetables because leaves with lesions were discarded (Zitter and Murphy, 2009). CMV had been identified as an economically important production constraint of field crops, vegetables and ornamentals due to its wider host range, widespread occurrence and the prevalence of CMV in the Pothwar region of Pakistan (Iqbal *et al.*, 2012; Ashfaq *et al.*, 2017). In addition to direct losses, the virus weakens plants, making them liable to other pest and diseases (Sharma, 2014). Most farmers are unaware of the symptoms of the virus infection and therefore do not recognize the disease, which means less or no adoption of control measures. Farmers need to be educated about the disease through extension programs; electronic and print media campaigns so that they know about it and adopt control measures. In the unmanaged areas, CMV may result in higher yield losses of up to 100%.

In addition to cucumber, melon, chilli and tomato which are known to be CMV natural hosts in Pakistan (Ali *et al.*, 2004; Akhtar *et al.*, 2008; Malik *et al.*, 2010; Iqbal *et al.*, 2012), CMV was also detected in pumpkin and bitter gourd for the first time in this study (Table 1). This indicates that the host range of CMV is increasing in the country. CMV detection from weed flora (Iqbal *et al.*, 2011) strengthens virus epidemiology. Farmers must therefore destroy weeds and volunteer plants in order to prevent epidemics of CMV. Some weeds intervene as the host of viruliferous aphids (Ruby *et al.*, 2011) and make CMV and other viral inocula capable of infecting newly planted crops.

Plant viruses produce variable symptoms depending on type of virus strain, host genotypes, age and time of infection of plants. CMV had three serotypes i.e. subgroup IA, IB and II, and the detection of subgroup IB and subgroup II in the Pothwar region of Pakistan through TAS ELISA affirms the results of Hosseinzadeh *et al.* (2012) who also used TAS ELISA for serotyping the prevalence of CMV. Subgroup I isolates usually predominate in tropical and subtropical zones, whereas subgroup II predominates in temperate regions (Hord *et al.*, 2001). In the Pothwar region subgroup II is more widely identified than subgroup I as the Pothwar region is located at the foothills of the Himalayas which have a temperate climate.

Closely related symptoms may result in misdiagnosis, particularly when farmers and researchers are relying merely on symptoms caused by CMV (Phan *et al.*, 2014). This thus emphasizes the importance of robust CMV detection and diagnostic methods. Through DAS-ELISA and RT-PCR followed by cloning and sequencing accurate detection of CMV can be achieved in samples without discrete symptoms. The accurate detection by RT-PCR of CMV shows the significance of this methodology for the diagnosis of viruses. The sequences of Pakistani CMV isolates were highly homologous to each other and to the CP gene of CMV isolates from other parts of the world within the same subgroup. Previous studies showed a high degree of CP gene sequence identity of CMV isolates of the same subgroup sampled from a single crop (Ahsan and Ashfaq, 2018), as well as of its global populations (Roossinck *et al.*, 1999; Takanami *et al.*, 1999). The fact that Pakistani CMV isolates were not all 100% identical to each other indicates a certain degree of CMV diversity in the country. The phylogenetic relationship revealed that the subgroup IB isolates clustered with the isolates from India and Egypt. Sister clad with Egyptian isolate confirms the presence of genetic variation in Pakistani isolates as they show a relationship with an isolate of a different geographical location. Similarly, Pakistani subgroup II isolates clustered with isolates of UK, USA, Japan and Hungary. The clustering of CMV isolates based on subgroups and formation of clusters with representative isolates has also been reported by many scientists (Roossinck *et al.*, 1999; Ohshima *et al.*, 2016).

Mutation, re-assortment and recombination are the main causes of genetic diversity in RNA viruses (Holmes, 2006; Akinyemi *et al.*, 2016) which may result in the enclosure of

discrete sequence components, along with interchange, repetition or obliteration of existing viral elements. Recombination is useful to understand viral evolution, and host adaptation in the production and elimination of variations in viral sequences. Furthermore, many researchers have reported that the rate of spontaneous mutations in plant RNA viruses is quite lower than their animal counterparts (Tromas and Elena, 2010). The recombination events in some CMV isolates are in the agreement of previous reports of confirmed mutations and genetic re-assortment (Roossinck, 2002; Lin *et al.*, 2004). Furthermore, In the present study recombination breakpoint events between the 370th and 630th nucleotide by four statistical methods implemented in the RDP4 program were detected in Pakistani isolate AAICu (MH119070) that is probably recombinant between the Indian isolate X89652 and the Pakistani isolate AARTF (MH119068) which served as minor and major parents, respectively.

The dispersion and coverage of the virus population perceived in this study require proper and continuous surveillance of the disease spread in symptomatic plants and other crops grown in a cropping pattern and effective management. In addition, a rapid and effective detection method, which doesn't entail prior data about the virus, should also be used to detect viruses, i.e. next-generation sequencing. Phylogenetic analysis, bioinformatics and deep sequencing as well as a comparison of the various species of viruses found in vegetables, demonstrate significant virus molecular variability that causes serious losses. Moreover, the spread of new genetic types indicates a great threat to crops, which should be tackled using effective viral diagnostic and management methods.

***Conclusions*:** The widespread occurrence of CMV in 10 cucurbit and solanaceous crops in all sampling sites of the Pothwar region with overall 20.98% disease incidence in 2016-17 highlights the necessity of regular disease surveys to be carried out for timely detection of new diseased hosts. This would make it possible to develop and implement disease management strategies earlier. The presence of CMV in newly infected hosts, relatively higher disease incidence and an increasing number of subgroup II isolates in succeeding years suggest an alarming situation for future successful production of vegetable crops. Moreover, recombination detection will force us to devise breeding programs in such a way that new resistant varieties will withstand recombinant strains. There is a need to educate farmers about CMVD to increase their knowledge, awareness and control of diseases which will directly have a fruitful effect on crop yield and quality.

***Acknowledgements*:** Funding for this study was provided by the Higher Education Commission of Pakistan under the Indigenous 5000 PhD Fellowship Program Phase-II, Batch-II, vide Pin No. 213-64590-2AV2-047.

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Received 28 May 2019; Accepted 6 Jul 2020; Published (online) 1 Sept 2020]