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Reverse Transcription-polymerase Chain Reaction (RT-PCR) Based Detection and Identification of Monopartite *Bean Common Mosaic Virus* in Mungbean

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ABSTRACT

Background: *Bean common mosaic virus* (BCMV) which belongs to the genus *Potyvirus* and the family *Potyviridae*, causes significant yield losses in legumes across the globe. Traditional methods for BCMV detection and diagnosis, such as visual inspection and serological assays, are often time-consuming, labour-intensive and may lack the sensitivity required for early detection. Advances in molecular techniques have made RT-PCR a powerful tool for the accurate detection of BCMV-infected plants.

Methods: Three pairs of specific primers that flank the BCMV coat protein and polyprotein gene were designed using the Primer 3web tool to amplify fragments approximately ranging from 191 to 205 bp. The RT-PCR protocol was then standardized, including the optimization of annealing temperatures for all three primers, followed by the sequencing of the amplified PCR products. The, specificity and sensitivity of primer pair BCMV2 were then established.

Result: Three pairs of specific primers were designed and RT-PCR protocol was standardized. The sequenced PCR products showed 98.03% to 99.50 % nucleotide similarity with the corresponding sequences of BCMV isolates used in primer designing. Further, the BCMV2 primer pair specifically amplified the target amplicon (200 bp) from the suspected leaf sample and no amplicon was observed when tested against other pathotypes of potyvirus such as BCMNV, BYMV, CABMV, SMV and PeMoV. The designed primer was sensitive enough to detect 0.05% of target cDNA. Additionally, the RT-PCR protocol was validated using field samples collected from Hyderabad, India. ELISA in combination with RT-PCR using the developed specific BCMV primer pair will ensure a foolproof, sensitive and rapid, procedure for diagnosis of BCMV in the quarantine processing of imported germplasm.

Key words: BCMV, BCMV-specific primers, Detection, Mungbean, RT-PCR, Sensitivity, Specificity.

INTRODUCTION

Bean common mosaic virus (BCMV) is distributed worldwide and causes significant yield losses (50%-100%) in the host crop plants (Drijfhout, 1991; Sastry and Zitter, 2013; Deepika *et al*., 2023) and belongs to the genus *Potyvirus,* family *Potyviridae* (Barnett *et al*., 1992). Potyvirus is a single stranded, positive sense RNA (Genome size~10 kb). BCMV is a non-enveloped, flexuous rod-shaped particle made up of approximately 2000 CP monomers encapsidating the linear, positive-sense, single-stranded RNA genome of ~9.9 kbp (Dougherty and Carrington 1988; Urcuqui-Inchima *et al.,* 2001; Wylie *et al.,* 2017). It measures about 750 nm×15 nm (Urcuqui-Inchima *et al.,* 2001). BCMV first reported in 1917 from the USA on common bean (*Phaseolus vulgaris*), is one of the earliest described viruses affecting economically important pulses (Stewart and Reddick, 1917) and initially named as bean mosaic virus. Later to differentiate it from Bean yellow mosaic virus (BYMV), the epithet common was added (Pierce, 1934). It can infect 94 plant species of which 83 of which are leguminous making it one of most after devastating viruses that infect legumes. In nature BCMV predominantly occurs on *Phaseolus vulgaris* (Zaumeyer and Thomas, 1957; Drijfhout and Bos, 1977). Worrall *et al*., (2019) states that, BCMV has wide range of hosts; different strains of BCMV are found to be infectious in different legume crops such as *Vigna radiata* (Kaiser and Mossahebi, 1974), *Crotalaria striata* (Sarkar and Kulastreshtha, 1978), *Arachis hypogaea*

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(Demski *et al.,* 1984) *V. unguiculata* subsp. *unguiculata* (Purciful and Gonsalves, 1985; Abhishek *et al*., 2024), *Glycine max* L. (Zhou *et al.,* 2014), *V. angularis* (Li *et al.,* 2016). Wild legume hosts like *V. trilobata* and *Macroptiliuma tropurpureum* were also infected by BCMV (Coutts *et al.,* 2011; Worrall *et al.,* 2019). The genetic diversity of BCMV and host specificities of its isolates were found to be generally correlated (Gibbs *et al.,* 2008; Zhou *et al.,* 2014; Li *et al.,* 2016), resulting in distinct phylogenetic clades corresponding to several species of legumes with associated systemic infection of the virus.

BCMV is a seed borne virus (Pandey and Parmar, 2023) and becomes more serious as it spreads through various species of aphids in non-persistent manner (Kapil *et al.,* 2011). Thus, even if it is carried through seed at a very low rate, its secondary spread can occur due to the activities of various aphid species active during the crop season. The deleterious effects of infection not only diminish seed quality but also reduce yield due to reduction in number of pod formation (66%) as well as loss in seed yield of 68% (Hampton, 1975). Depending on the cultivar, BCMV infection known to generate three types of symptoms *viz*., mosaic, systemic necrosis (black rot) and hypersensitive reaction. In general, BCMV infected plants exhibits mottling, curling, blistering, stunting and malformation of the primary leaves and mosaic of trifoliate leaves. Infected plants bear under-developed pods and delays seed maturity.

In India, different strains of BCMV are known to occur and reported to cause yield losses ranging from 35-98% across various host and leading for significant economic losses to the country (Nalini *et al*., 2006; Singh and Schwartz, 2010; Sastry and Zitter, 2013; Deepika *et al.*, 2023; Li *et al.,* 2014; Abhishek *et al*., 2024). In Latin America, Schwartz and Galvez (1980) reported complete yield loss due to BCMV. The invention of the thermal cycler and polymerase chain reaction has made these techniques indispensable and essential virus detection/diagnosis in most plant virus diagnostic laboratories, mainly due to their simplicity and robustness. Molecular tools like reverse transcription-polymerase chain reaction (RT-PCR) is a reliable powerful method for accurate detection of plant viruses, particlularly those with RNA genomes. RT-PCR has been used the after for rapid and sensitive detection of plant viruses as reported by previous researchers Gibbs and Mackenzie 1997; Hampton 1975; Langeveld *et al.,* 1991; Marie Jeanne *et al.,* 2000; Trajkova and Khristova, 2008; Musavi, 2013) including BCMV (Udayashankar *et al.,* 2012; Manjunatha *et al*., 2017; Abhishek *et al*., 2024) in common bean, cowpea and black gram. Hence, this study was conducted with the objective of developing RT-PCR protocol for the detection of mungbean infecting RNA virus BCMV.

MATERIALS AND METHODS

Plant sample and RNA extraction

The total RNA was isolated from the ELISA positive BCMV infected leaf samples collected from ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR) Regional Station, Hyderabad, India using RNeasy® Plant Mini Kit (QIAGEN® kit catalogue Nos.74903) according to the manufacturer's instructions. The experiment was conducted during the 2018-2019, in the Virology lab, Department of Plant Quarantine, ICAR-NBPGR, New Delhi.

cDNA synthesis

The Thermo Scientific Verso cDNA Synthesis kit was used to synthesise cDNA according to the manufacturer's protocol. RT-PCR was performed following the method of Ryu and Park (1995). In brief, a total of 20 µl reaction mixture was prepared by combining the followig ingredients such as 4 µl of 5X cDNA synthesis buffer, 2 µl of dNTP mix (Conc), 1 µl of Random hexamer RNA primer, 1 µl of RT enhancer,

Table 1: Details of source of isolate, GenBank accession number, crop from which it was isolated and country of origin.

Primer name	Source of isolate	GenBank accession number	Crop	Country of origin
BCMV1	CD011	KM051425	*Glycine max*	China
	CD021	KM051426	*Glycine max*	China
	CD025	KM051427	*Glycine max*	China
	CD026	KM051428	*Glycine max*	China
	CD030	KM051429	*Glycine max*	China
BCMV2	Viva2	MH024839	*Phaseolus vulgaris*	United States of America
	313615	MH024840	*Phaseolus vulgaris*	United States of America
	PG1	MH024838	*Phaseolus vulgaris*	United States of America
	3915	MH024842	*Phaseolus vulgaris*	United States of America
	RU1-CA	MH024843	*Phaseolus vulgaris*	United States of America
BCMV3	DXH023	KJ807810	*Glycine max*	China
	DXH024	KJ807811	*Glycine max*	China
	DXH025	KJ807812	*Glycine max*	China
	PStV-JX014	KJ807813	*Glycine max*	China
	HZZB007	KJ807814	*Glycine max*	China

1 µl of Verso enzyme mix (units), 4 µl of viral RNA (template RNA, 100 ng) and nuclease free water to make up the volume. The mixture was reverse transcribed at 42°C for 30 min and the RT enzyme was inactivated at 95°C for 2 min. The cDNA thus obtained was used for standardization of PCR.

Primer design

To design specific primers for BCMV detection, the complete genome sequences of BCMV isolates available at NCBI were utilized. Table 1 provides the details on the source of isolates, GenBank accession number, the crop from which it is isolated and the country of origin for all three primers.

The complete BCMV genome sequences were retrieved and aligned using ClustalX2 software. GeneDoc software was used to clean up the sequences. Primers were designed using Primer3 web tool. Three pairs of gene specific primers that flanked the poly protein and coat protein gene were designed. Primers BCMV1, BCMV2 and BCMV3 were expected to amplify fragments 205 bp, 200 bp and 191 bp respectively. Thermodynamics and secondary structures of the primers were assessed using Mfold web tool. Primer specificity was checked using NCBI BLAST analysis tool.

Standardization of RT-PCR

The obtained cDNA was subjected to PCR in a programmable DNA thermal cycler (Perkin Elmer Cetus, model 480) for 40 cycles (94°C 30 s, 51-60°C 20 s and 72°C 45 s) after an initial denaturation at 95°C for 4 min. After the 40th cycle, the final extension was for 7 min at 72°C. To determine the optimum annealing temperature for the primer and template, several temperatures (51-60°C) were examined using gradient PCR for all three pairs of primers (BCMV1, BCMV2 and BCMV3). PCR amplifications were conducted in a thermocycler in 20 µl reaction mixture containing 5 µl of template cDNA, 10 µl of Go Taq® Master Mix (2X) (Promega, Madison WI USA), 1 µl each of forward and reverse primers(5 µM) and nuclease-free water to make up the volume. Amplified PCR products of 10 µl aliquot from each reaction mixture were analysed by electrophoresis on a 1.0% agarose gel in TAE buffer (Sambrook *et al.,* 1989). The amplified DNA from primers, BCMV1, BCMV2 and BCMV3 was sequenced (Sequencher Tech, Ahmadabad) and sequence similarity were analysed using NCBI-BLAST to confirm target specificity.

Specificity of primer set BCMV2

The specificity of primer set BCMV2 was validated in silico against NCBI database and in *vivo* against infected plant positive control panel of Potyviruses. The panel included *Bean common mosaic necrosis virus* (BCMNV), *Bean yellow mosaic virus* (BYMV), *Cowpea aphid-borne mosaic virus* (CABMV), *Soybean mosaic virus* (SMV) and *Peanut mottle virus* (PeMoV) and are known to infect legumes. Standardized RT-PCR protocol as mentioned in above section.

Sensitivity of primer set BCMV2

The cDNA (200 ng/µl) from BCMV infected leaf tissue was serially diluted and used as template in sensitivity assays. The serially diluted cDNA obtained was subjected to PCR amplification using BCMV2 primer set as described. The amplified DNA fragments were electrophoresed in 1.2% agarose gel and analysed.

Validation of RT-PCR protocol

Virus infected mungbean leaf samples (Nine) collected from Hyderabad, were tested for BCMV by RT-PCR with BCMV2 primers. RT-PCR was performed in a thermal cycler as described and the resultant products were analysed on a 1.5% agarose gel (Fig 6).

RESULTS AND DISCUSSION

Optimisation of RT-PCR - Primer designing

The first step in standardiing the RT-PCR protocol is primer design. After checking the thermodynamic properties and secondary structure of primers using Mfold software three primer pairs (Table 2) were selected which are the most conserved and recognises the coat protein gene and polyprotein gene of BCMV.

Standardization of PCR protocol

Viral RNA isolated from the infected leaf sample was reverse transcribed. The cDNA was amplified at 10 different annealing temperature (51.3 to 60.8°C) in gradient PCR using primers mentioned. The amplified products of gradient PCR were analysed in 1.2% agarose gel by electrophoresis amplification occurred at all ten annealing temperature and sharp bands were observed at 51.3°C for all three primers (BCMV1, BCMV2 and BCMV3) (Fig 1, Fig 2 and Fig 3).

Sequencing analysis

The PCR products were directly sequenced and generated the expected amplicons of 203 bp, 200 bp and 184 bp DNA

Table 2: List of BCMV specific primers designed in this study.

Primers	Name	Sequences	Product size	No of bases
BCMV1	Forward primer	5′CAACACTCCGCCAGATCATG 3′	205 bp	20
	Reverse primer	5′ GCTGCCTTCATCTGTGCTAC 3′		20
BCMV2	Forward primer	5′AAAGCCAACACTCCGCAAAT 3′	200 bp	20
	Reverse primer	5′ GCTGCCTTCATCTGTGCTAC 3′		20
BCMV3	Forward primer	5′ GTAGCACAGATGAAGGCAGC 3′	191 bp	20
	Reverse primer	5′ CTATTCAGCGACGCGAGATG 3′		20

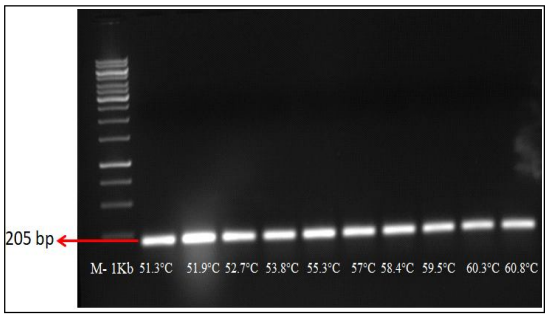


Fig 1: Gel picture showing gradient PCR based amplification of BCMV infected samples using BCMV 1 primer with a temperature range of 51.3°C-60.8°C.

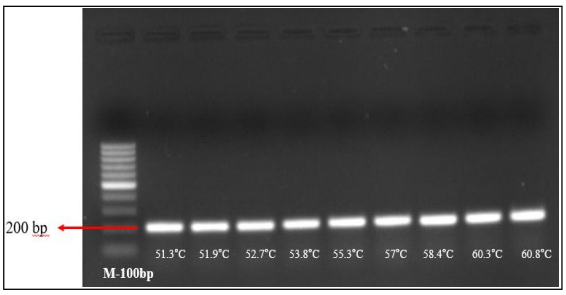


Fig 2: Gel picture showing gradient PCR of based amplification of BCMV infected samples using BCMV 2 primer with a temperature range of 51.3°C-60.8°C.

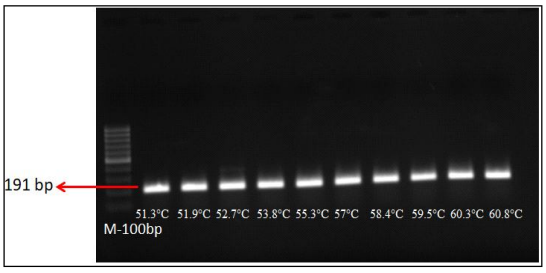


Fig 3: Gel picture showing gradient PCR based amplification of BCMV infected samples using BCMV 3 primer with a temperature range of 51.3°C-60.8°C.

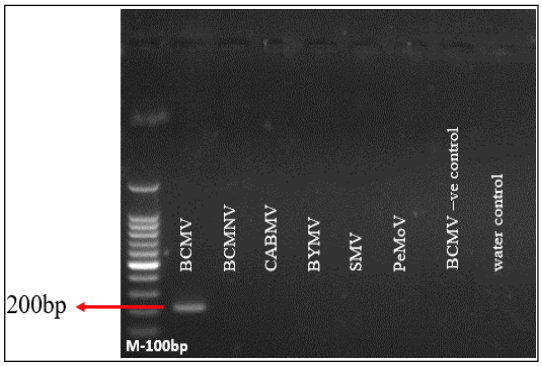


Fig 4: Gel picture showing specificity of primer set BCMV 2 in amplifying different potyviruses infecting legumes.

fragments respectively. Consensus sequences were used for sequence analysis and the coding region was identified using the software NCBI-ORF Finder. The sequences were analysed for nucleotide similarity with BCMV sequences available in nucleotide database using BLAST search of GenBank and showed 98.03%, 99.50% and 98.91% nucleotide similarity respectively with corresponding sequences of BCMV isolates used in primer design. Apart from that, the consensus sequences were matched with the coat protein gene and polyprotein gene of BCMV and confirming specificity to BCMV.

Specificity of primer set BCMV2

The specificity of primer sets BCMV1, BCMV2 and BCMV3 was validated *in silico* against the NCBI database. For *in vivo* specificity test, BCMV2 was tested against a panel of legume between infecting potyviruses namely, BCMNV, BYMV, CABMV, SMV and PeMoV and no cross between reactivity was observed (Fig 4). Thus, the specificity of designed primer was revalidated and can be used for specific BCMV detection.

Sensitivity of primer set BCMV2

The cDNA (200 ng/µl) from BCMV between infected leaf tissue was diluted to various concentration (100%, 50%, 20%, 10%, 1%, 0.1% and 0.05%) and used for sensitivity assays using BCMV2 primer set. The assay was sensitive enough to detect as little as 0.05% (0.10 ng/µl) cDNA of BCMV from infected plants (Fig 5). In validation using

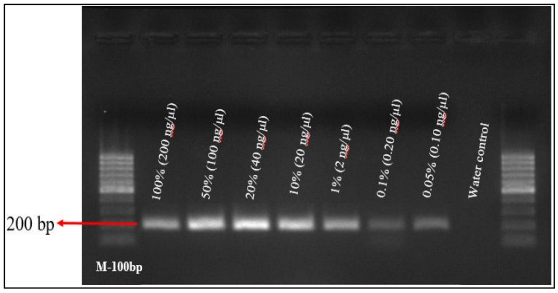
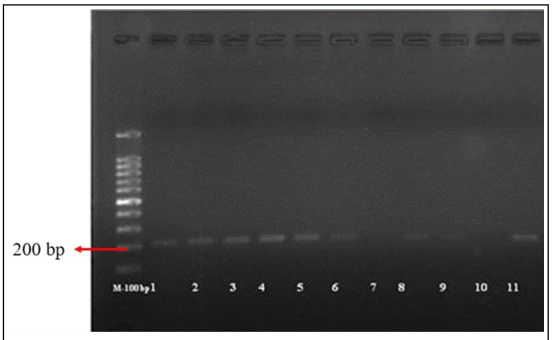


Fig 5: Gel picture showing sensitivity of primer set BCMV 2 with diluted BCMV cDNA.



(M-100bp Ladder; 1 to 11-Field samples of cowpea).Indicate the amplicon size in the figure.

Fig 6: Screening cowpea samples for validation of primer BCMV 2.

cowpea samples collected from Hyderabad, the primer set BCMV2 detected BCMV in 9 samples (Fig 6).

This indicates that the designed primer pair BCMV2 is specific. BCMV can be easily detected by the RT-PCR with these primers and the same PCR conditions can be used for detecting BCMV in mungbean and different kinds of pulses. Several papers have reported genus-specific primers of plant viruses including *potyviruses*, *cucumoviruses*, *tospoviruses*, *closteroviruses* and *luteoviruses* (Choi *et al.,* 1999; Gibbs and Mackenzie, 1997; Karasev *et al.,* 1994; Mumford *et al.,* 1996; Robertson *et al.,* 1991). To our knowledge, this is the first report of the BCMV-specific primers for the RT-PCR. Therefore, RT-PCR using this set of primers targeting the coat protein gene will be very useful for the detection of BCMV. This indicates that the RT-PCR with the designed set of primers is useful for reliable and rapid detection of BCMV in imported germplasm both in seed and leaf samples. Moreover, it might allow future discovery of potential new strains of this virus.

CONCLUSION

In our study, we successfully developed and validated a sensitive and specific RT-PCR-based method for detecting Bean Common Mosaic Virus (BCMV), a major threat to mungbean and other pulse crops. Unlike traditional symptom-based detection, which is often unreliable due to the similar symptoms produced by different viruses or varied symptoms from a single virus, RT-PCR provides highly accurate results. We standardized three species-specific primer pairs targeting the BCMV coat protein and polyprotein genes and optimized the RT-PCR conditions. Notably, the BCMV2 primer pair demonstrated robust performance, consistently amplifying a 200 bp target with 99.50% nucleotide similarity to BCMV isolates and showing no cross-reactivity with other potyvirus pathotypes in in vivo specificity tests. It was also capable of detecting virus concentrations as low as 0.05%. This developed RT-PCR protocol is a rapid and reliable approach for BCMV detection and can be routinely used to aid in intercepting BCMV in imported germplasm during quarantine procedures.

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Authors contribution

VCC-Conceptualization of research, review and editing of the manuscript. DDD-Designing experiment, methodology, investigation and original draft preparation KT-Critical inputs for experimentations, review and editing of the manuscript. GJAAK-Methodology and investigation. KK-Methodology and investigation, sequence submission and manuscript editing. VDS-Methodology and investigation. BP-Review and editing of manuscript. PKHR-Review of manuscript. The authors read and approved the final manuscript.

Ethical approval

Not applicable.

Statement of funding

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Conflict of interest

The authors declare no conflict of interest.

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