



RT-PCR Assay Standardization for the Diagnosis of Cowpea Infecting *Bean Common Mosaic Virus*

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ABSTRACT

Background: Bean common mosaic disease (BCMD) caused by *Bean common mosaic virus* (BCMV) poses a significant threat to legume crops worldwide. BCMV, a monopartite potyvirus, has been a concern since its detection in 1917 due to its seed-borne nature. Symptoms include mosaic patterns, necrosis, stunting and yield reductions, leading to substantial economic losses in affected regions. Reverse transcription-polymerase chain reaction (RT-PCR) offers a precise and efficient method for BCMV detection, necessitating the development of specific primers for accurate diagnosis.

Methods: Specific primers (BCMV-polyp1, BCMV-coatp2 and BCMV-polyp3) were designed by aligning BCMV genome sequences and employing Primer3 software. RNA isolation from infected samples and cDNA synthesis were performed using standard protocols. RT-PCR was conducted with various annealing temperatures to optimize conditions. Primer specificity of BCMV-coatp2 primer set was confirmed *via* BLAST analysis and *in vivo* testing against closely related potyviruses. Sensitivity assays were carried out using serially diluted cDNA samples from infected tissue.

Result: The designed primers demonstrated high specificity to BCMV, with no cross-reactivity observed against closely related potyviruses. Optimization of RT-PCR conditions revealed distinct bands at an annealing temperature of 51.9°C for all primer sets. Sequencing confirmed amplicon sizes (175 bp, 157 bp and 189 bp) and alignment with BCMV sequences. Sensitivity analysis showed the assay capable of detecting BCMV at concentrations as low as 0.14 ng/μl. Validation with field samples confirmed the effectiveness of the primers in identifying BCMV-infected plants.

Key words: BCMV, Cowpea, Potyvirus, RT-PCR optimization, Sensitivity, Specificity.

INTRODUCTION

Legumes represent sustainable dietary staples that particularly fulfill the essential nutritional need for protein among the global human population. The production and productivity of legumes are frequently hampered by various biotic and abiotic stresses. Among these challenges, Bean common mosaic disease stands out as a major concern. This disease is caused by *bean common mosaic virus* (BCMV), which is a monopartite *potyvirus*, a single-stranded, positive-sense RNA virus with a genome size of 10 kb. It has dimensions of approximately 750 nm in length and 15 nm in width (Urcuqui-Inchima, 2001). It is classified under the genus *Potyvirus* within the family *Potyviridae* (Worrall *et al.*, 2019; Wylie *et al.*, 2017, Abhishek *et al.*, 2024). In nature BCMV predominantly occurs on *P. vulgaris* (Zaumeyer and Thomas, 1957; Drijfhout and Bos, 1977).

Since its initial detection in 1917 on French bean in the USA, BCMV has subsequently spread to numerous countries, primarily due to its seed-borne characteristics (Morales and Bos, 1988; Tang and Feng, 2022). It is seed-transmitted, can remain viable in bean seeds for over three decades serving as a major source of infection and as it spreads through different aphid species the infection may get severe (Zaumeyer and Thomas, 1957; Kapil *et al.*, 2011; Sastry, 2013; Aishwarya *et al.*, 2020). The harmful impacts of infection extend beyond just diminishing seed quality; they also result in a 66% reduction in the pods number and a 68% decrease in seed yield. Even symptomless infection

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can have the yield (Hampton, 1975; Morales, 2006). In India, various strains of BCMV have been identified and documented to result in yield reductions (ranging from 35% to 98%) across a wide array of hosts, leading to significant economic losses for the country (Sastry, 2013; Dinesh *et al* 2018; Manjunatha *et al.*, 2017; Aishwarya *et al.*, 2020; Deepika *et al.*, 2023).

According to Worrall *et al.* (2019), BCMV demonstrates a wide hosts range, with various strains infecting different

species. Remarkably, BCMV can infect over 100 species across 44 genera, including adzuki bean, common bean, cowpea, groundnut, lablab bean, mung bean, sesamum, soybean, siratro (*Macroptilium atropurpureum*), *V. trilobata* and so on (Yan *et al.*, 2009; Li *et al.*, 2016; Worrall *et al.*, 2019; Yadav *et al.*, 2021). Reports indicate that 36 aphid species from 21 genera, such as *Aphis craccivora*, *Acyrtosiphon pisum*, *Hyalopterus atriplicis*, *Myzus persica*, *Macrosiphum ambrosiae* and *Aphis fabae* are effective vectors in transmitting BCMV (Edwardson *et al.*, 1984; Kuhn *et al.*, 1989; Kachroo *et al.*, 2020).

Symptoms primarily result from three factors: the host plants themselves, prevailing environmental conditions and the specific viral strains involved. The symptom comprises of a mosaic of well-defined dark green island patches, often observed in a vein-banding pattern. Furthermore, it also shows necrosis of upper parts and followed by death of plant (Grogan and Walker, 1948; Wu *et al.*, 2018). However, the virus can also induce other symptoms such as mosaic, mottling, blistering, leaf curl, chlorosis, dwarfing, underdeveloped or no pod formation in infected plants (Feng *et al.*, 2019; Deepika *et al.*, 2023). Polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR) are effective methods for identifying viral infections, such as the DNA virus MYMIV and the RNA viruses PBNV and BCMV, by targeting their coat proteins (Biswas *et al.*, 2009; Chalam *et al.*, 2024). RT-PCR, a molecular technique, is particularly dependable and powerful for accurately detecting plant viruses, especially since most of these viruses have RNA genomes.

RT-PCR detection of BCMV offers significant advantages over traditional methods in terms of sensitivity, specificity, speed, quantification, automation, robustness and cost-effectiveness, making it the method of choice for many plant virus diagnostics. Previous researchers used RT-PCR for the detection of plant viruses including BCMV (Trajkova and Khristova, 2008; Udayashankar *et al.*, 2012; Musavi, 2013; El-Sawy *et al.*, 2014; Manjunatha *et al.*, 2017;

Deepika *et al.*, 2023; Iqbal *et al.*, 2021). Therefore, this research was undertaken with the aim of standardizing RT-PCR protocol specifically designed for the identification of the potyvirus BCMV.

MATERIALS AND METHODS

Designing primers for RT-PCR amplification

The present research was carried out in the Virology Lab of the Division of Plant Quarantine, ICAR-National Bureau of Plant Genetic Resources, New Delhi, during 2019-2020. To develop specific primers for detecting BCMV, complete genome sequences of BCMV isolates from the NCBI database were utilized (Table 1). The complete BCMV genome sequences were initially aligned using ClustalX2 software. The sequences were then refined with the help of GeneDoc software. Primer design was accomplished using the Primer3 web tool, resulting in the generation of three pairs of gene-specific primers flanking the polyprotein and coat protein gene regions. These primers, named BCMV-polyp1, BCMV-coatp2 and BCMV-polyp3, were anticipated to amplify fragments of 175 bp, 157 bp and 189 bp, respectively. The thermodynamic properties as well as secondary structures of the primers were evaluated via the Mfold web tool, while primer specificity was confirmed through NCBI Blast analysis.

RNA Isolation and cDNA synthesis for RT-PCR detection of BCMV

Using RNeasy® Plant Mini Kit (QUIAGEN® kit catalogue Nos.74903 and 74904), complete RNA extraction from BCMV infected (ELISA positive) and healthy samples (ELISA negative) of cowpea collected from the polyhouse has been completed (Fig 1A and B). The Thermo Scientific Verso cDNA Synthesis kit was utilized for cDNA synthesis, following the method described by Ryu and Park (1995). In a 20 µl reaction mixture, 1 µl of verso enzyme mix (units), 1 µl of RT enhancer, 1 µl of random hexamer, 2 µl of dNTP mix (Conc), 4 µl of 5X cDNA synthesis buffer and 4 µl of viral

Table 1: Source, GenBank accession number, isolate name, crop and country of origin of isolates used for primer development.

Primer name	GenBank accession number	Source of isolate	Crop name	Country of origin
BCMV-polyp1	KJ807801	CD012	<i>Glycine max</i>	China
	KJ807802	CD023	<i>Glycine max</i>	China
	KJ807803	DXH005	<i>Glycine max</i>	China
	KJ807804	DXH008	<i>Glycine max</i>	China
	KJ807805	DXH015	<i>Glycine max</i>	China
BCMV-coatp2	MF498886	Ir-GoB	<i>Phaseolus vulgaris</i>	Iran
	MF498887	Ir-MJB	<i>Phaseolus vulgaris</i>	Iran
	MF498888	Ir-MS1	<i>Vigna unguiculata</i>	Iran
	MF498889	Ir-MS2	<i>Vigna unguiculata</i>	Iran
BCMV- polyp3	KJ807816	HZZB012	<i>Glycine max</i>	China
	KJ807817	NKY017	<i>Glycine max</i>	China
	KJ807818	NKY019	<i>Glycine max</i>	China
	KJ807819	NKY021	<i>Glycine max</i>	China
	KJ807820	NKY022	<i>Glycine max</i>	China

RNA (template RNA, 100 ng) were combined, with nuclease-free water added to achieve the desired volume. The mixture underwent reverse transcription at 42°C for 30 minutes, followed by inactivation of the RT enzyme at 95°C for 2 minutes. The resulting cDNA was then utilized for PCR standardization.

Optimization of RT-PCR conditions

The cDNA obtained was amplified through PCR in a thermal cycler (Perkin Elmer Cetus, model 480) for 40 cycles, starting with an initial denaturation at 95°C for 4 minutes. Each cycle comprised denaturation at 94°C for 30 seconds, annealing at temperatures ranging from 51 to 60°C for 20 seconds and extension at 72°C for 45 seconds. Following the 40th cycle, a final extension step was conducted at 72°C for 7 minutes. To determine the optimal annealing temperature for the primers and template, a gradient PCR was performed across different temperatures (51-60°C) for all three pairs of primers (BCMV-polyp1, BCMV-coatp2 and BCMV-polyp3).

PCR amplifications were carried out in a 20 µl reaction mixture containing 5 µl of template cDNA, 1 µl each of forward and reverse primers (5 µM), 10 µl of Go Taq® Master Mix (2X) (Promega, Madison WI USA) and nuclease-free water to reach the final volume. Subsequently, 10 µl aliquots of the amplified PCR products from each reaction mixture were subjected to electrophoresis in a 1.0% agarose gel using TAE buffer (Sambrook *et al.*, 1989). The amplified DNA fragments produced by the BCMV-polyp1, BCMV-coatp2 and BCMV-polyp3 primers were submitted for sequencing (Sequencher Tech, Ahmadabad) and the similarity of the sequences was analyzed using NCBI-BLAST to confirm target specificity.

Assessment of specificity and sensitivity of primer set BCMV-coatp2

The specificity of primer set BCMV-coatp2 was validated *in silico* against the NCBI database and *in vivo* against infected plant positive control panel of Potyviruses. The panel included Bean common mosaic necrosis virus (BCMN), 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.

The cDNA synthesized from BCMV-infected cowpea leaf tissue, with a concentration of 290 ng/microliter, underwent serial dilution and served as the template for sensitivity assays. The diluted cDNA samples were then subjected to PCR amplification using the BCMV-coatp2 primer set, following the procedure outlined as above. Subsequently, the resulting amplified DNA fragments were separated by electrophoresis in a 1.2% agarose gel and gel images were captured by using a gel documentation system (Alpha Imager1, USA).

Validation of RT-PCR protocol

Virus infected cowpea leaf samples (Nine) collected from ICAR- NBPGR Field (Fig 2A-C), were tested for BCMV by RT-PCR with BCMV-coatp2 primers. RT-PCR was performed in thermal cycler as described above and the resultant products were analyzed on a 1.2% agarose gel.

RESULTS AND DISCUSSION

Optimization of RT-PCR Protocol: Primer design and standardization

The initial stage in standardizing the RT-PCR protocol involves primer design. BCMV sequences sourced from GenBank *via* NCBI were aligned using ClustalX2 software. Subsequently, GeneDoc software was utilized to refine the sequences, selecting consensus sequences for further analysis. Primers were then designed using Primer3 software, resulting in multiple primer candidates. Following this, the thermodynamic properties and secondary structures of the primers were assessed using Mfold software. From the pool of candidate primers, three primer pairs were chosen (Table 2). The nucleotide specificity of these primers was verified using the NCBI BLAST tool.

The viral RNA extracted from the infected leaf sample underwent reverse transcription to cDNA *via* RT-PCR. Subsequently, the cDNA was subjected to amplification at 10 varying annealing temperatures ranging from 51 to 60°C using the aforementioned primer set in a gradient PCR setup. The resulting amplified products from the gradient



Fig 1 A and B: BCMV-infected cowpea plants in a polyhouse.

PCR were analyzed *via* electrophoresis in a 1.2% agarose gel. Among the 10 different annealing temperatures tested, distinct and well-defined bands were observed consistently at 51.9°C for all three primer sets (Fig 3-5).

The BCMV-polyp1 primer yielded a DNA fragment with a size of 175 bp, while BCMV-coatp2 produced a fragment of 157 bp and BCMV-polyp3 resulted in a fragment of 189 bp. Subsequently, the PCR products were subjected to direct sequencing, confirming the presence of the expected amplicons with sizes of 175 bp, 157 bp and 189 bp, respectively.

The sequenced fragments were compared to sequences in the nucleotide database using BLAST. With primer pair BCMV-polyp1, the amplicon aligned with the polyprotein gene of BCMV, showing 92.75% nucleotide similarity. Primer pair BCMV-coatp2 matched the coat protein gene, exhibiting 90.68% similarity. Primer pair BCMV-polyp3 also aligned with the polyprotein gene, with 92.5% similarity. Thus, consensus sequences matched both the coat protein gene and polyprotein gene of BCMV, confirming specificity to BCMV.

Specificity and sensitivity assessment of primer set BCMV-coatp2

The specificity of primer set BCMV-coatp2 was confirmed both through *in-silico* analysis against the NCBI database and *in-vivo* testing using infected plant positive controls. In the *in-silico* analysis against the NCBI database, the amplicon generated by the primer pair BCMV-coatp2 showed that the location of amplified region in the genome is matching with the coat protein gene of BCMV and 90.68%

nucleotide similarity with BCMV (Table 3; Fig 6). We also evaluated the *in-silico* specificity of primer pairs BCMV-polyp1 and BCMV-polyp3. For BCMV-polyp1, the amplicon aligned with the polyprotein gene, showing 92.75% similarity. Similarly, BCMV-polyp3 matched the polyprotein gene, demonstrating 92.5% similarity.

In-vivo specificity testing of BCMV-coatp2 included a panel consisting of BCMNV, BYMV, CABMV, SMV and PeMoV, all belonging to the *Potyvirus* genus and known to infect legumes. No cross-reactivity was detected during specificity testing against this panel, as illustrated in Fig 7. The cDNA (290 ng/μl) from BCMV-infected leaf tissue was serially diluted and utilized for sensitivity testing. These diluted cDNA samples underwent PCR amplification employing the BCMV-coatp2 primer set. The sensitivity analysis revealed the capability of the assay to detect concentrations as low as 0.14 ng/μl (Fig 8). During validation with cowpea samples obtained from ICAR-NBPGR, the BCMV-coatp2 primer set identified BCMV in 9 samples (Fig 9). Thus, the designed primers are accurate to detect and identify BCMV, a monopartite potyvirus.

Our research demonstrates that the designed primer pairs BCMV-polyp1, BCMV-coatp2 and BCMV-polyp3 exhibit specificity. BCMV can be readily identified through RT-PCR using these primers and the same PCR conditions can be applied for detecting BCMV in various types of legumes. In consistent with our results, previous researchers also optimized PCR conditions for the detection of BCMV (Chiquito-Almanza *et al.* 2017). Several workers developed genus specific primers such as *cucumoviruses*,



Fig 2 A-C: BCMV-infected cowpea plants in a polyhouse.

Table 2: List of species-specific primers designed for BCMV.

Primer pair	Name	Sequences	Product size	Melting temperature	Base pairs number
BCMV-polyp1	FP	5' ACAATC AGG ACC TGG ACA GC 3'	175 bp	60.12°C	20
	RP	5' GGAACC TGCATT CAC ATC CT 3'		59.93°C	20
BCMV-coatp2	FP	5' TGG AAT CTG GGA AGG ACAAG 3'	157 bp	60.04°C	20
	RP	5' TTT TGA AGC CGA GGAACA AC 3'		60.23°C	20
BCMV-polyp3	FP	5' ACAATC AGG ACC TGG ACA GC 3'	189 bp	60.12°C	20
	RP	5' ACAACC TTC CCT TTG GAA CC 3'		60.20°C	20

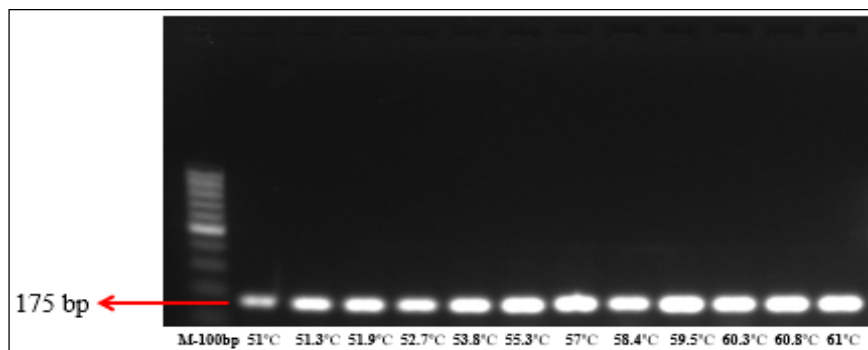


Fig 3: Gradient PCR using BCMV-polyp1 primer set with a temperature range of 51°C-60°C.

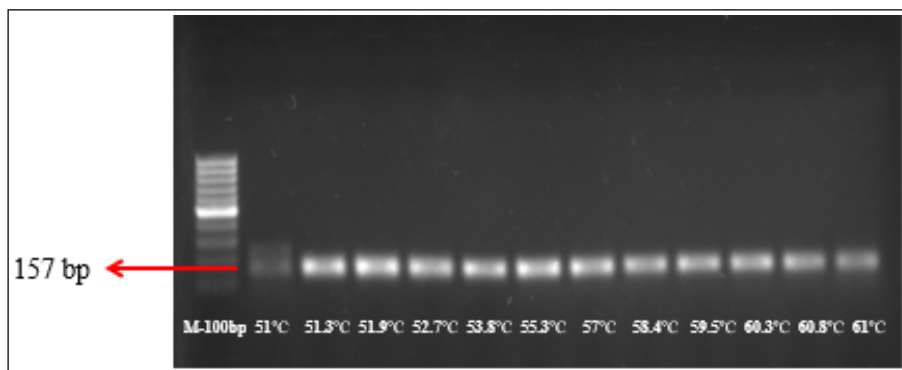


Fig 4: Gradient PCR using BCMV-coatp2 primer set with a temperature range of 51°C-60°C.

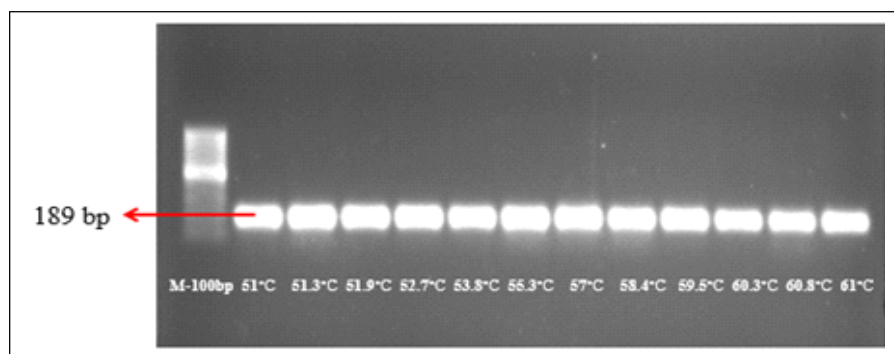


Fig 5: Gradient PCR using BCMV-polyp3 primer with a temperature range of 51°C-60°C.

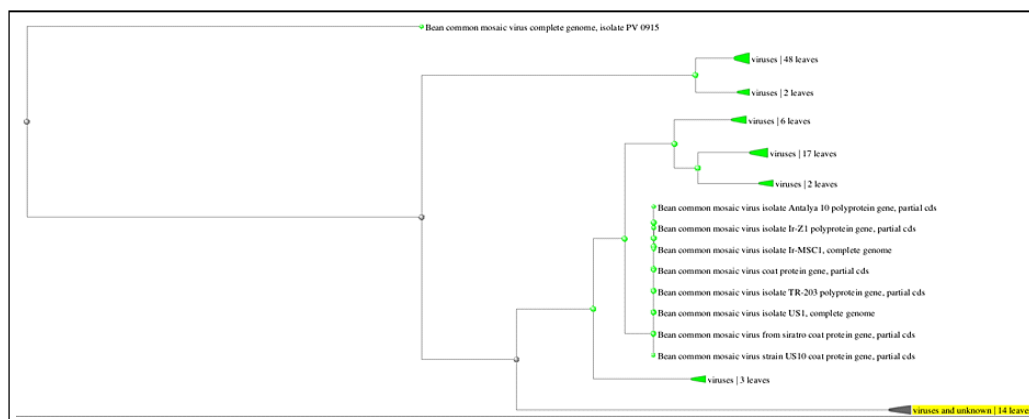


Fig 6: Dendrogram of nucleotide sequence amplified with the BCMV-coatp2 primer pair based on BLAST search results.

potyviruses, *luteoviruses*, *tospoviruses*, *Comovirus* and *closteroviruses* (Choi *et al.*, 1999; Gibbs and Mackenzie, 1997; Karasev *et al.*, 1994; Mumford *et al.*, 1996; Nito *et al.*, 2024; Robertson *et al.*, 1991). Thus, RT-PCR utilizing this primer set, which targets the coat protein gene, proves highly beneficial for BCMV detection. This suggests that the RT-PCR assay employing the designed primer set is effective

for the accurate and prompt detection of BCMV in imported germplasm, both in seed and leaf samples. Additionally, it may facilitate the identification of potential new strains of this virus in the future. Overall, the developed RT-PCR protocol utilizing the BCMV-polyp1, BCMV-coatp2 and BCMV-polyp3 primer sets are reliable and efficient method for the detection and identification of BCMV, a monopartite potyvirus. Its

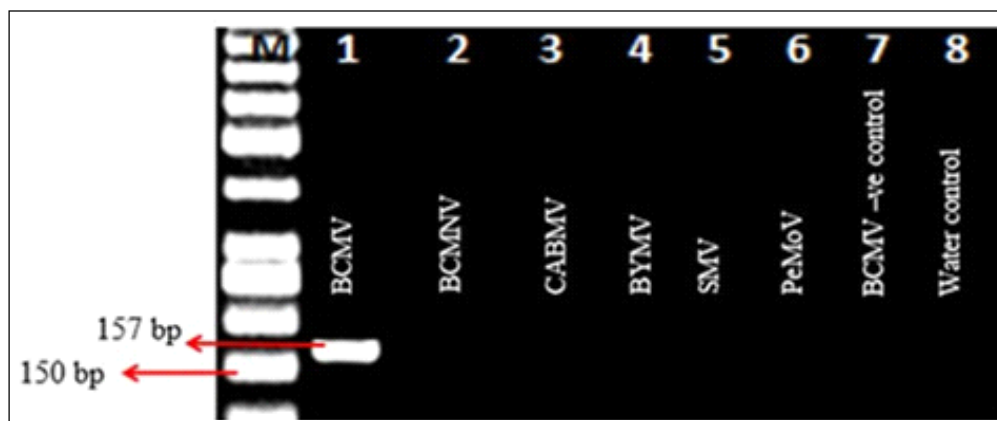


Fig 7: Gel image showing specificity of primer set BCMV-coatp2.

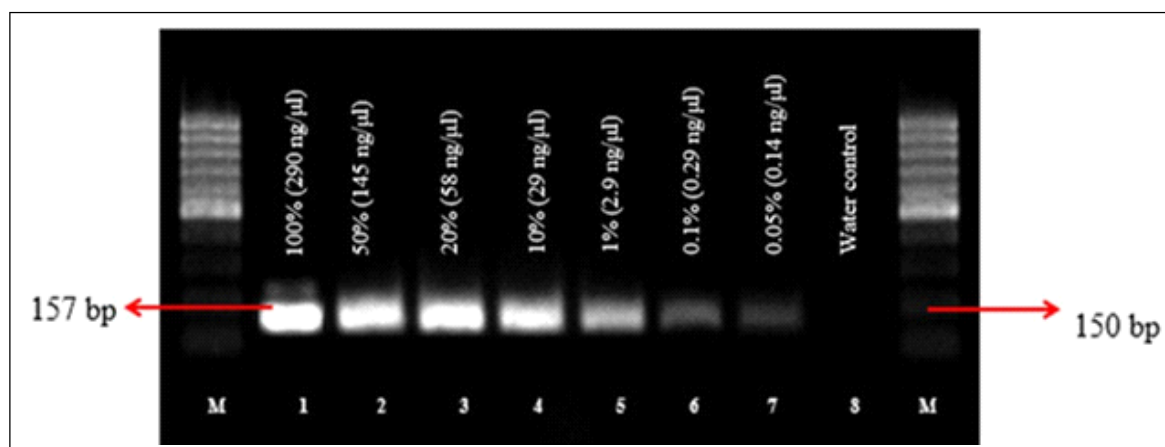


Fig 8: Gel image showing sensitivity of primer set BCMV-coatp2.

Table 3: BLAST search results for amplicon with BCMV-coatp2 primer pair.

Description	Accession no.	Query cover	E value	Per cent identity
Bean common mosaic virus strain Blackeye isolate BF-E142 coat protein gene, partial cds	MF277042.1	85%	3e-34	90.68%
Bean common mosaic virus strain Blackeye isolate BF-E138 coat protein gene, partial cds	MF277040.1	85%	3e-34	90.68%
Bean common mosaic virus strain Blackeye isolate BF-E2 coat protein gene, partial cds	MF277032.1	85%	3e-34	90.68%
Bean common mosaic virus isolate soybean, complete genome	KC832501.1	85%	3e-34	90.68%

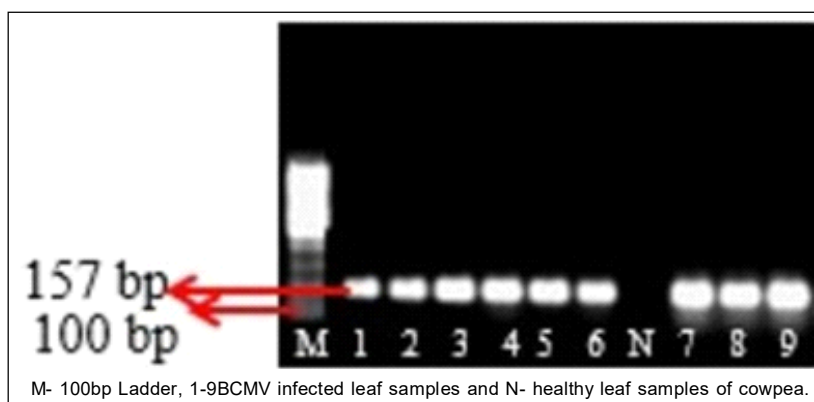


Fig 9: Screening cowpea samples for validation of primer BCMV-coatp2.

specificity, sensitivity and accuracy make it suitable for both research and practical applications in monitoring and managing BCMV infections in leguminous crops.

CONCLUSION

Our study successfully designed and established the specificity using three pair of specific primers, BCMV-polyp1, BCMV-coatp2 and BCMV-polyp3 and validated BCMV-coatp2 for the detection of BCMV using RT-PCR. These primers demonstrated high specificity to BCMV, allowing for accurate detection across various leguminous hosts. The optimized RT-PCR protocol exhibited robustness and sensitivity, capable of detecting BCMV at low concentrations. The developed assay offers significant advantages over traditional methods in terms of speed, specificity and cost-effectiveness, making it a valuable tool for BCMV diagnostics in its identification, quarantine inspection, disease free conservation of seeds. Overall, the RT-PCR protocol utilizing the BCMV-polyp1, BCMV-coatp2 and BCMV-polyp3, primer sets provide reliable and efficient means of detecting and identifying BCMV, thereby contributing to the management of this detrimental virus in leguminous crops.

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Conflict of interest

The authors declare no conflict of interest.

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