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RESEARCH ARTICLE - BEES

Nucleic Acid-based Identification of Major Honeybee (*Apis mellifera* L.) Viruses in the Jammu Region of India

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Abstract

The present investigation was conducted from 2019 to 2020 to study the incidence pattern and molecular diagnosis of major honeybee (*Apis mellifera* L.) viral diseases in different areas of Jammu region of Jammu and Kashmir (UT). Using the RT-PCR technology, research was done to confirm that *A. mellifera* is infected by the Israeli acute paralysis virus (IAPV), the Thai sacbrood virus (TSBV), the Kashmir bee virus (KBV), and sacbrood virus (SBV). Particular primers for distinct viruses verified the existence of the virus in chosen samples from various areas, including Jammu, Samba, Ramban, and Udhampur. The samples taken from all four districts were found to contain illness. Clear and distinct bands with molecular sizes of roughly 110, 122, and 119 bp for IAPV, KBV, and SBV were formed by primer pairs of various viruses. The four districts' samples contained no evidence of the Thai sacbrood virus. The incidence range of Israeli acute paralysis disease was 8.33 to 21.42 %. The incidence range for Kashmir bee disease was 5.71 to 28.33 %. The incidence range for sacbrood disease was 8.0–18.55%. In the apiary at SKUAST-J, Chatha, the incidence ranges for IAPV, KBV, and SBV diseases were 0 to 6.25, 0 to 4.16, and 0 to 9.43 %, respectively.

Introduction

The European honey bee, or *Apis mellifera,* is an extremely important species for agriculture, the environment, and the economy. Acute bee paralysis virus (ABPV) (Bailey et al., 1963; Govan et al., 2000) and deformed wing virus (DWV) (Martin et al., 2012) seem to be the most likely causes of bee deaths. While Israeli acute paralysis virus (IAPV) was initially identified as a predictor of colony losses in the United States (Cox-Foster et al., 2007), later investigations suggest that the virus was merely one of several potential causes of losses (de Miranda et al., 2010). In the field, the diagnosis of viral infections is made by looking at the brood combs and looking for sick larvae. However, a definite diagnosis might be challenging because the symptoms of viral diseases can be easily confused with other illnesses or anomalies in the brood. Therefore, confirmatory laboratory tests like ELISA and PCR (Polymerase Chain Reaction) are employed to confirm that the sacbrood virus is present in suspected colonies. Detection of honey bee viruses has been done through several methods, including the immune gel diffusion technique (Allen & Ball, 1996), electron microscopy (Rana & Rana, 2015), enhanced chemiluminescent western blotting (Allen et al., 1986), and reverse transcriptase (Rana et al., 2011). Research is also being conducted on the molecular characterization of honey bee viruses through the RT-PCR technique in different parts of the world (Grabensteiner et al., 2001; Rana et al., 2011).

Various diseases, predators, and pests impair the health and normal working of *A. mellifera* is one of the major problems among beekeepers in Jammu and Kashmir.

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Knowledge about the seasonal incidence of various diseases and enemies of *A. mellifera* under changing scenarios is scarce. A qualitative survey of pests and pathogens of A. mellifera is needed under different conditions. Further, prospective beekeepers should be aware of various limiting factors to protect honey bee colonies and get maximum benefit from beekeeping ventures.

Materials and Methods

A. mellifera virus-infected larvae and adults were sampled from private beekeepers' apiaries in the specified districts (Jammu, Samba, Ramban, and Udhampur) as well as from the apiary at SKUAST-J University (Plate 1). We obtained 200 samples, with 50 samples collected from each district. Plastic vials were used for sample collection and placed in iceboxes to preserve RNA integrity during transportation to the laboratory for subsequent PCR analysis.

For RNA isolation, glassware and plasticware were treated with di-ethyl pyrocarbonate (DEPC) water. RNA was isolated from honey bee samples using the Trizol method. Samples were crushed using liquid nitrogen and mixed with 500 µl of Tri-Reagent (Sigma-Aldric). After centrifugation, the supernatant was transferred to a microcentrifuge tube, and RNA was bound to a silica pad in an RNA binding column (Zymo Research, CA, USA). The bound RNA was eluted and stored at -20 °C.

Utilizing the Hi-cDNA Synthesis Kit (HI MEDIA), cDNA synthesis was initiated by selecting a tube and adding either Random Hexamer or Oligo (dT), followed by an RNA template and Molecular Biology Grade Water for PCR. After 5-minute incubation at 65 °C and rapid cooling, reverse transcription reagents were added according to the manufacturer's instructions.

The cDNA synthesis mixture included Template RNA Primer Mixture, RT Buffer for MMuLV, 10X Solution for MMuLV, M-MuLV Reverse Transcriptase, 10mM dNTP mix, and Molecular Biology Grade Water. As indicated in the provided protocol, the mixture was gently mixed, centrifuged, and subjected to thermal cycling.

Primer set for KBV and IAPV

Primer Name	Primer Sequence (5'- 3')	Length (bp)	Melting temperature (°C)
KBV3CPRTFW	CAAACTGCTGAATCAATGTCAAAAT	25	50
KBV3CPRTRV	ACATGCCTCTACTTTGTCACATTCA	25	50
IAPVVP3RTFW	CGAACTTGGTGACTTGAAGG	20	50
IAPVVP3RTRV	GCATCAGTCGTCTTCCAGGT	20	50

Primer set for SBV and TSBV

Primer Name	Primer Sequence (5'- 3')	Length (bp)	Melting temperature (°C)
TSBVRTFW	AGGGAAATTACTAATATACCTTGCCTTCATATA	33	57
TSBVRTRV	GGCAACTATGGCACTCTCACCATAGTT	27	60
SACP1RTFW	GGACTCTTATACCGATTTGTTTAATGGTTGGG	32	61
SACP1RTRV	CGCGTCTAACATTCCAGATTCTTCGTCC	28	61

RT-PCR reactions were performed using a CFX Connect Real-Time thermal cycler machine. Each reaction contained a cDNA template, SYBR Green PCR Master Mix, forward and reverse primers and nuclease-free water. Reactions were run in duplicate with a specific thermal profile.

After amplification, the RT-PCR products were stained with ethidium bromide, put onto a 2% agarose gel, electrophoresed, and seen under a UV transilluminator.

Results and Discussion

The primer pair IAPVVP3RTFW/IAPVVP3RTRV amplified the VP3 region present in the IAPV genome. The amplicons (amplified product) were observed under UV-transiluminator. IAPV-infected samples 2 and 4 (Chatha), 5 (Dalwah), 6 (Purmandal), 7 (R. S. Pura), 8 and 9 (Sudhmahadev) yielded distinct and clear bands of molecular size approximately 110 bp when the primer pair IAPVVP3RTFW/IAPVVP3RTRV was used (Plate 2).

The primer pair KBV3CPRTFW/KBV3CPRTRV amplified the 3C-pro region present in the KBV genome. The amplicons (amplified product) were observed under UV-transiluminator. The primer pair KBV3CPRTFW/ KBV3CPRTRV produced clear and distinct bands of molecular size of approximately 122 bp from KBV infected samples 4 (Chatha), 5 (Vijaypur), and 6 (Dabrah) (Plate 3).

The primer pair SACP1RTFW/SACP1RTRV amplified the polyprotein region present in the SBV genome.

The amplicons (amplified product) were observed under UV-transiluminator. The primer pair SACP1RTFW/SAC P1RTRV produced clear and distinct bands of molecular size of approximately 119 bp from SBV-infected samples 1 (Chatha), 2 (Sudhmahadev), and 3 (Digdol) (Plate 3).

The primer pair TSBVRTFW/TSBVRTRV did not amplify any region in the TSBV genome.

The present study finds support from Francis and Kryger (2012), who used RT-PCR techniques for identifying the Kashmir bee virus (KBV) and Israeli acute bee Paralysis virus (IAPV) of honey bees (*Apis mellifera* L.). Specific RT-PCR product lengths were KBV 414bp and IAPV 138bp.

Rana et al. (2011), who studied the detection of sacbrood virus using ELISA and RT-PCR techniques in diseased prepupae of *A. mellifera* obtained from Himachal Pradesh, India, also support the current study. The sacbrood virus was verified using three primers: Sb1f/r, Sb3 f/r and Sb5 f/r. The genome was amplified to create discrete bands with anticipated molecular sizes, roughly 487, 267, and 597 bp.

The incidence of Israeli acute paralysis disease caused by the Israeli acute paralysis virus was recorded with a minimum of 8.33% in the Vijaypur colonies of Samba and a maximum of 21.42% in Sudhmahadev colonies of Udhampur. Similarly, data was recorded on Kashmir bee disease caused by the Kashmir bee virus and sacbrood disease caused by sacbrood virus in *A. Mellifera* is given in Table 1 and Fig 1. The incidence of Kashmir bee disease was recorded with a minimum of 5.71% in Chatha colonies of Jammu and a maximum of 28.33% in Vijaypur colonies of Samba. The incidence of Sacbrood disease was recorded with a minimum of 8.0% in the Digdol colonies of Ramban and a maximum of 18.75% in the Dalwah colonies of Ramban.

The incidence of Thai sacbrood disease caused by the virus was not recorded during 2019-2020 in any studied regions.

Table 1. Incidence of IAPV, KBV, SBV and TSBV diseases in the different areas of Jammu region.

District	Location	Number of apiaries surveyed	Number of colonies per apiary	Number of infected colonies				% colony infection			
				IAPV*	KBV[#]	SBV[$]	TSBV[&]	IAPV	KBV	SBV	TSBV
Jammu	Chatha	1	70	7	4	11	0	10.00	5.71	15.71	0
	R. S. Pura	1	60	10	14	6	0	16.66	23.33	10.0	0
Ramban	Digdol	1	150	17	11	12	0	11.33	7.33	8.0	0
	Dalwah	1	80	15	17	15	0	18.75	21.25	18.75	0
Samba	Purmandal	1	80	7	14	7	0	8.75	17.50	8.75	0
	Vijaypur	1	60	5	17	8	0	8.33	28.33	13.33	0
Udhampur	Sudhmahadev	1	70	15	11	10	0	21.42	15.71	14.28	0
	Dabrah	1	30	4	5	5	0	13.33	16.66	16.66	0
Total		**8**	**600**	**80**	**94**	**73**					

*IAPV - Israeli acute paralysis virus
[#]KBV - Kashmir bee virus
[$]SBV - Sacbrood virus
[&]TSBV - Thai sacbrood virus

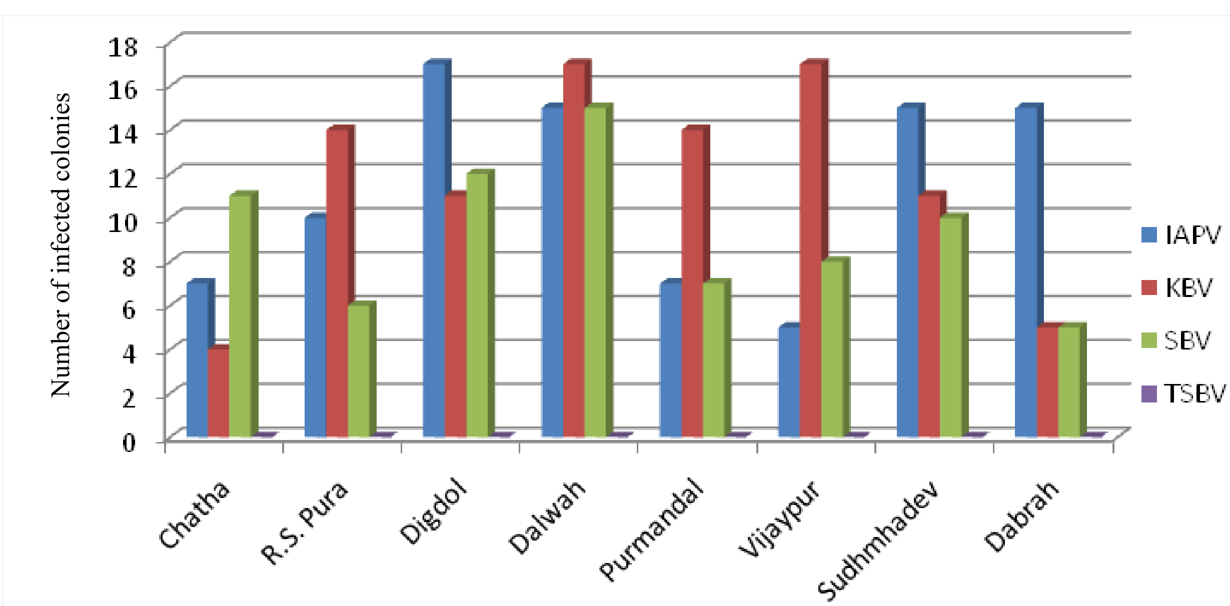

Fig 1. Incidence of IAPV, KBV, SBV and TSBV diseases in the different areas of Jammu region.

IAPV - Israeli acute paralysis virus
KBV - Kashmir bee virus
SBV - Sacbrood virus
TSBV - Thai sacbrood virus

In the present study, it was observed that the incidence of Israeli acute paralysis disease was recorded with a minimum of 8.33% in Vijaypur and a maximum of 21.42% in Sudhmahadev. The incidence of Kashmir bee disease was recorded with a minimum of 5.71 % in Chatha and a maximum of 28.33% in Vijaypur. The incidence of sacbrood disease was recorded with a minimum of 8.0% in Digdol and a maximum of 18.75% in Dalwah. The incidence of Thai sacbrood disease was not recorded during 2019-2020 in any of the surveyed regions. Rana and Rana (2015) reported that the virus killed the honey bee brood at the prepupal stage (10 days of age) on the second day after the sealing of the brood. Two sites in Himachal Pradesh's Solan district, Nauni, were the sites of SBV detections. From March to May, colonies infected between 0.39 and 5.20 percent of the brood during the spring and summer. In the spring and summer (March to June) of the second location, in the Kangra district of Jachh (Himachal Pradesh), the disease was likewise found to infect 0.23% to 2.1% of the brood. It was discovered that there was a strong correlation between disease occurrence and brood raising.

The data recorded on the incidence of viral diseases in *A. mellifera* from July 2019 to April 2020 at SKUAST-J apiary is given in Table 2 and Fig 2. The incidence of Israeli acute paralysis disease was found to maximum in October (6.25%) and minimum in November to February (0%). Kashmir bee disease was found to be the most common in September (4.16%), while no incidence was recorded in surveyed apiaries in December and January. Similarly, the incidence of sacbrood disease was found to be maximum in March (9.43%) and minimum in December to January (0%).

Table 2. Incidence of IAPV, KBV, SBV and TSBV diseases in *Apis mellifera* in the University apiary.

Months	Number of colonies	Number of infected colonies				% colony infection in the apiary			
		IAPV*	KBV#	SBV$	TSBV&	IAPV	KBV	SBV	TSBV
July	48	1	1	2	0	2.08	2.08	4.16	0
August	48	2	1	1	0	4.16	2.08	2.08	0
September	48	2	2	2	0	4.16	4.16	4.16	0
October	48	3	1	3	0	6.25	2.08	6.25	0
November	48	0	1	1	0	0	2.08	2.08	0
December	48	0	0	0	0	0	0	0	0
January	48	0	0	0	0	0	0	0	0
February	48	0	1	1	0	0	2.08	2.08	0
March	53	1	2	5	0	1.88	3.77	9.43	0
April	53	2	1	3	0	3.77	1.88	5.66	0

*IAPV- Israeli acute paralysis virus
#KBV- Kashmir bee virus
$SBV- Sacbrood virus
&TSBV- Thai sacbrood virus

The incidence of Thai sacbrood disease was not recorded, as given in Table 2 and Fig 2.

The present study observed viral disease incidence in SKUAST-J apiary from July 2019 to April 2020. The incidence of Kashmir bee disease was found to be maximum in September (4.16%) and minimum in December to January (0%). The studies recorded the Israeli acute paralysis disease, Kashmir bee disease, sacbrood, and Thai sacbrood diseases for the first time from the Jammu region of Jammu and Kashmir. Acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Kashmir bee virus (KBV), and sacbrood virus (SBV) were also detected by Nielsen et al. (2008) using real-time polymerase chain reaction (RT-PCR). SBV was found in 78 apiaries, DWV in 55 apiaries, ABPV in 11 apiaries, CBPV in 4 apiaries, BQCV in 1, and KBV in 1 apiary among the six virus types they identified with considerable difference rates. The discussed results also corroborate the findings of a simulation published in 2007 by Todd et al., who investigated the virus status of honey bee colonies infected with *Varroa destructor*. Throughout the investigation, they saw the viruses CBPV, BQCV, SBV, DWV, and KBV, with DWV and KBV being the most prevalent.

In related research, Ai et al. (2012) found that seven honey bee viruses were prevalent in Chinese apiaries of *A. cerana* and *A. mellifera*. Six viruses were identified from *A. mellifera*, excluding KBV. DWV was discovered to be the most common, occurring in 9% of the apiaries. Within the apiaries, BQCV, SBV, IAPV, ABPV, and CBPV were found in 44%, 21%, 18%, 6%, and 9% of them, respectively.

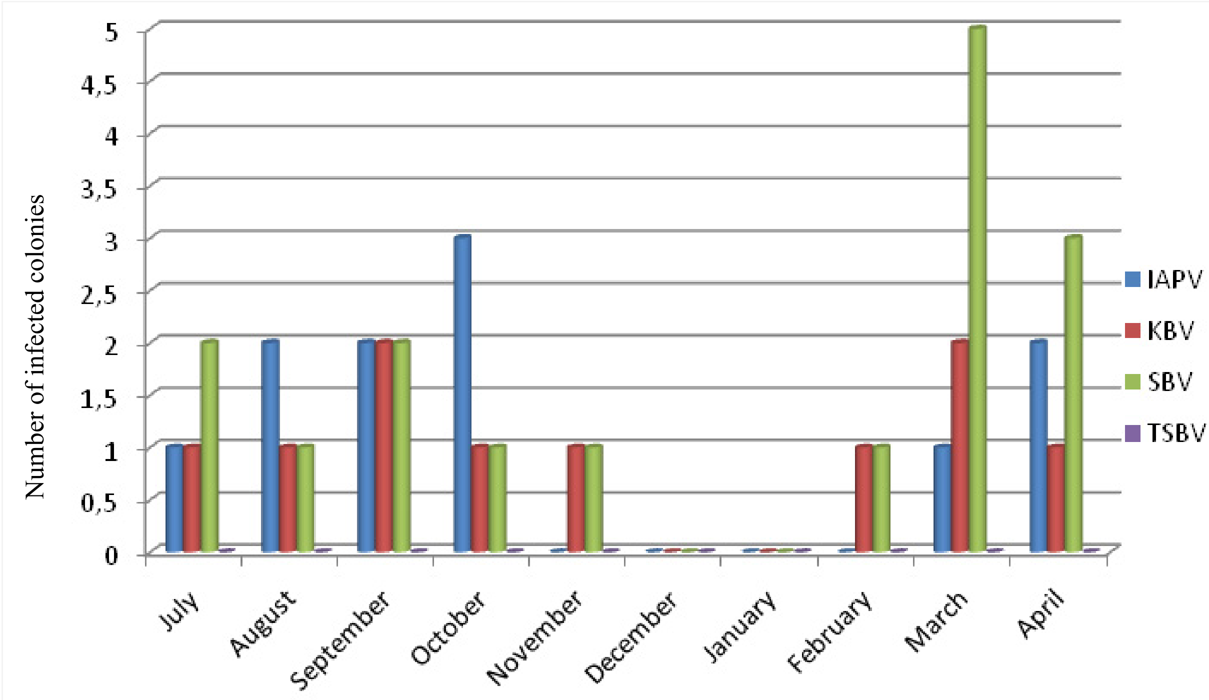

Fig 2. Incidence of IAPV, KBV, SBV and TSBV diseases in *Apis mellifera* during July 2019-April 2020 in the apiary at SKUAST-J, Chatha.

IAPV - Israeli acute paralysis virus
KBV - Kashmir bee virus
SBV - Sacbrood virus
TSBV - Thai sacbrood vírus

The prevalence of viruses in the *A. cerana* apiaries was significantly different. The most common type, SBV, was found in 86% of the apiaries. DWV was next, found in 64% of the apiaries, and IAPV, CBPV, or BQCV were found in 7% of the apiaries. From populations of *A. cerana*, neither KBV nor ABPV were found. None of the samples from the two Chinese species of honeybees had KBV. The prevalence and occurrence of the following viruses were documented by Rustemoglu et al. (2019): Israeli acute paralysis virus (IAPV), sacbrood virus (SBV), chronic bee paralysis virus (CBPV), Kashmir bee virus (KBV), and black queen cell virus (BQCV). RT-PCR was used to determine whether BQCV, DWV, SBV, and CBPV infections were present for the first time. The most common virus found in bee samples was BQCV, which was detected in 32% of samples. DWV, SBV, and CBPV were detected in less than 24% of asymptomatic samples, with 23, 12, and 9% infection ratios for each virus, respectively. None of the analyzed bee samples in this survey had KBV or IAPV. BQCV+DWV 7 (7.7%), BQCV+SBV 4 (4.4%), BQCV+CBPV 2 (2.2%), DWV+CBPV 2 (2.2%), DWV+SBV 1 (1.1%), and DWV+BQCV+SBV 1 (1.1%) were among the double and triple infections that were found.

Author's Contribution

RNS: Conceptualization, methodology, investigation, analysis, writing-initial draft, writing-review & editing, visualization.

DS and BB: Conceptualization, supervision, resources, investigation, visualization.

PKK and SKM: Writing-original draft, writing-review & editing.

Declaration

The authors declare no conflict of interest.

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Material Supplementary

Plate 1: Map showing sites selected for survey in Jammu region.

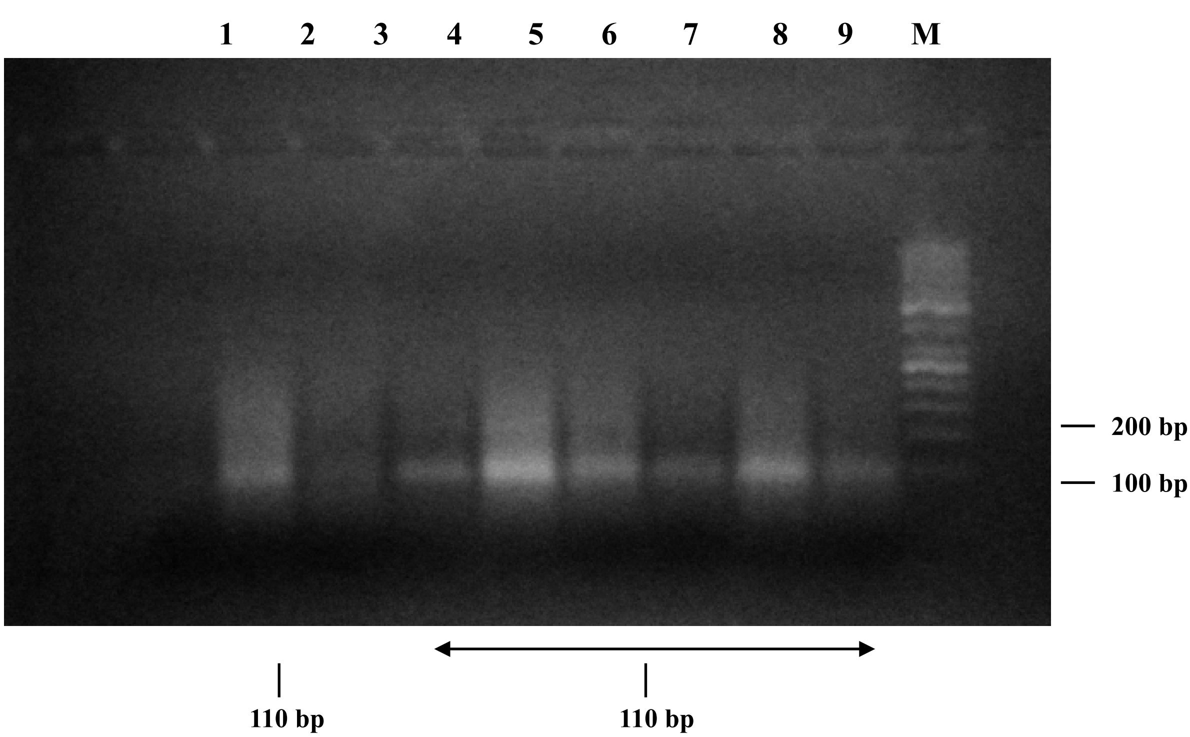

Plate 2: Bandingprofile of RT-PCR product using IAPV specific primer pair. M: (100 bp ladder), sample 2 and 4 (Chatha), 5 (Dalwah), 6 (Purmandal), 7 (R. S. Pura), 8 and 9 (Sudhmahadev): showing IAPV specific band (110 bp), 1 and 3: No amplification

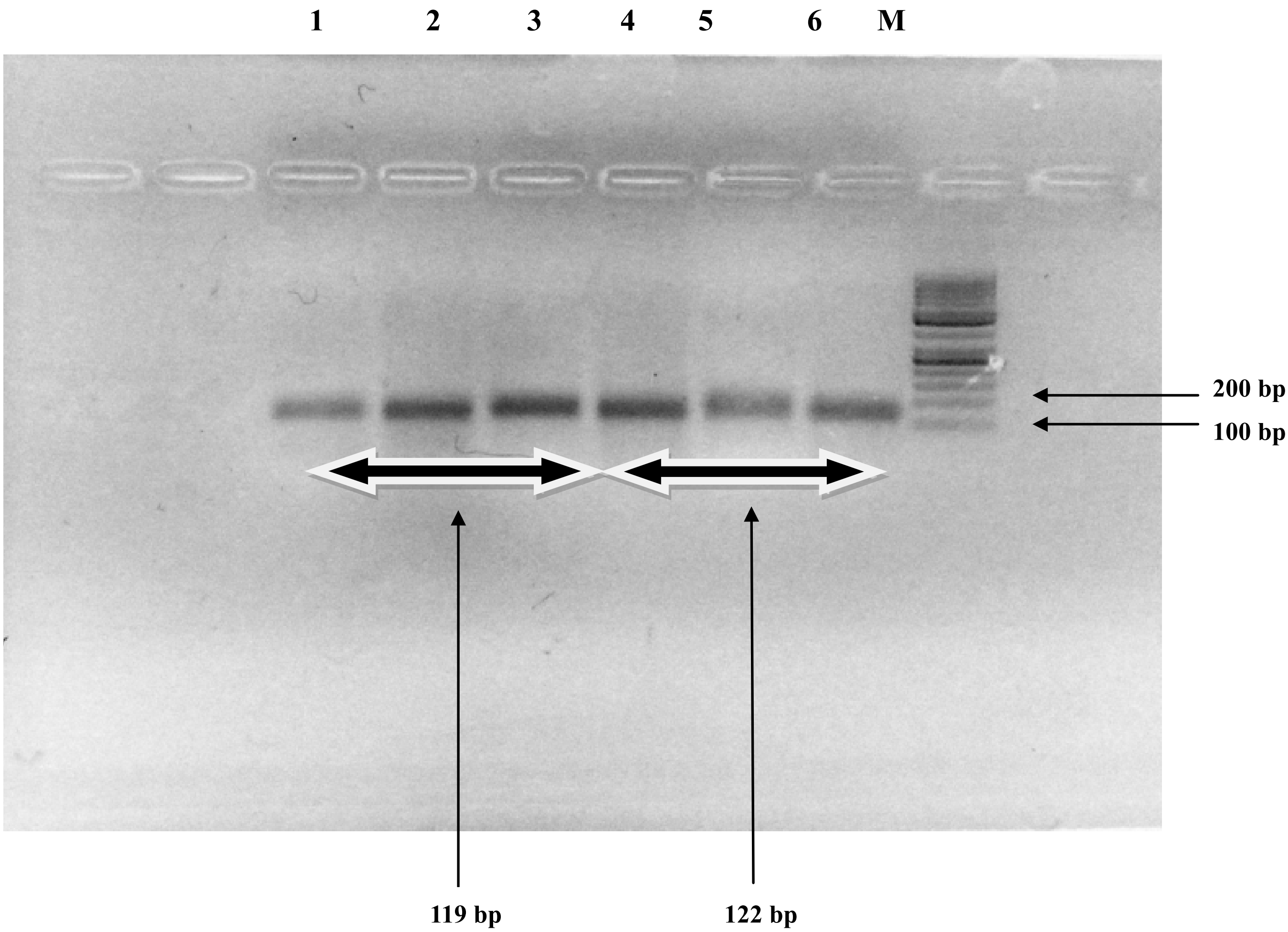

Plate 3: Bandingprofile of RT-PCR product using KBV and SBV specific primer pairs. M: (100 bp ladder), sample 1 (Chatha), 2 (Sudhmahadev), 3 (Digdol): showing SBV specific band (119 bp) and 4 (Chatha), 5 (Vijaypur) and 6 (Dabrah): showing KBV specific band (122 bp)