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DESIGNING OF RT-LAMP PRIMERS AND DETECTION OF SACBROOD VIRUS FROM INDIAN HONEY BEE *APIS CERANA INDICA* (F.)

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ABSTRACT

The present investigation was carried out on the sac brood virus (SBV) in *Apis cerana indica* of Tamil Nadu during 2018-19. Colonies were examined in major beekeeping regions, and diagnosis of SBV using specific primers was done to amplify the 834bp fragments through RT-PCR. Later these were compared with that of RT-LAMP (Reverse Transcriptase-Loop Mediated Isothermal Amplification), and a new set of primers were designed. In the RT-LAMP, the samples with SBV reacted with royal blue coloured hydroxyl naphthalene blue (HNB) dye and produced sky blue coloured fluorescence in positive reaction while the original royal blue colour was retained in the virus free samples. From among the cDNA isolated from twenty supposedly SBV infected samples, 10 and 8 samples were detected to have the viral cDNA by RT-PCR and RT-LAMP, respectively. RT-PCR is thus the best method for SBV diagnosis in the laboratory while the RT-LAMP is suitable for field level diagnosis.

Key words: *Apis cerana indica*, sac brood virus, Tamil Nadu, RT-PCR, RT-LAMP, hydroxyl naphthalene blue, field and laboratory diagnostics

The most serious viral disease of honey bees is sac brood virus (SBV). SBV is a single-stranded RNA virus (Iflavirus: Iflaviridae) with a worldwide distribution (Ghosh et al., 1999). The virus seriously reduces production of honey as well as other hive products in major beekeeping areas and also affects the pollination service in crop ecosystem (Pearce, 2014). SBV primarily infects young larvae and fails into pupae. The infected larvae turn white to pale yellow and then die (Bailey, 1975). Currently 22 viruses have been identified in honey bees and their prevalence is seen throughout the world. Molecular techniques had been developed for detection of the honey bee viruses, that includes immuno diffusion techniques (Allen and Ball, 1996), enzyme-linked immune sorbent assay, electron microscopy, Western blotting (Allen et al., 1986), reverse transcriptase polymerase chain reaction (RT-PCR) (Benjeddou et al., 2001; Grabensteiner et al., 2000; Parrella et al., 2006; Celle et al., 2008; Blanchard et al. 2008; Boncristiani et al., 2009; Yan et al., 2009; Kukielka and Sanchez Vizcaino, 2009; Sanpa and Chantawannakul, 2009; Aruna et al., 2016), real-time RT-PCR (Chen et al., 2005; Blanchard et al., 2007; Kukielka et al., 2008; Kukielka and Sanchez Vizcaino, 2009) and ultra-rapid real time PCR (Yoo et al., 2012). These techniques are expensive and time consuming. A simple, cheap and quick method for diagnosis of the honey bee viruses is required.

RT-LAMP is an easy, relatively accurate, rapid method for detection of specific honey bee viruses and less expensive (Notomi et al., 2000; Ushikubo, 2004; Tomita et al., 2008; Yamazaki et al., 2008; Aruna et al., 2019). In South Indian conditions especially Tamil Nadu apiaries, AcSBV are a major threat causing heavy losses in *A. cerana indica* colonies. Rapid diagnosis is important in controlling the spread of disease to other colonies in the apiaries. Identification of virus is most important because, a single infected honey bee may have more than one virus or multiple viral infections viz., acute bee paralysis virus (Bailey et al., 1963), Kashmir bee virus (Bailey and Woods, 1977), deformed wing virus (Ball and Allen, 1988; Martin, 2001; Highfield et al., 2009) and black queen cell virus were also noticed by several researchers in various parts

of the country. Without proper detection of the virus, management of the disease will be a big challenge. Hence, the present study for the detection of SBV infecting Indian honey bee *Apis cerana indica* through Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Reverse Transcriptase-Loop Mediated Isothermal Amplification (RT-LAMP).

MATERIALS AND METHODS

During the survey AcSBV infected larval as well as pre adult stages were collected from six different locations covering four districts in major beekeeping regions of Tamil Nadu. At each location individual larvae and pre pupae sample were collected in separate Eppendorf® tubes containing 70% ethanol and stored in deep freezer at-80ºC till further use. Infected broods reveal larval death, with dead larvae seen with tip of the head capsule turned upwards, dead prepupae turned into sac like structure and the colour of the affected larvae changed from white to yellow or grey.

Total RNA isolation was done with Geneipure™ Kit, in which RNA was isolated from AcSBV in prepupal samples. The infected brood samples were ground in 600µl of lysis buffer and 600µl of β- mercaptoethanol. The lysate of the homogenized tissue sample was filtered through column provided in the kit. The column was then spun at 10000 rpm for 1 min. Absolute ethanol was added at the rate of 0.75 volumes to the flow through. The ethanolic lysate was loaded on Geneipure™ RNA column and spun at 10000 rpm for 15 sec. The column was washed with 500 µl of diluted wash buffer I and spun at 10000 rpm for 15 sec. Then the column was washed twice with 500µl of diluted wash buffer II and spun at 10000 rpm for 30 sec. Fifty µl of elution buffer was added to the column and spun at 10000 rpm for one min.

The concentration and quantity of the prepared total RNA were determined by spectrophotometric analysis and agarose gel electrophoresis. The RNA (10 to 50 fold according to the expected yield) was diluted in TE buffer (10 mM Tris- HCl, 1 mM EDTA, pH 8) and measured using Bio Spectrophotometer, Germany. The quantity of RNA was analyzed on 1% agarose gel containing ethidium bromide at 55 volts for one hour and visualized under UV light and the gel was documented using gel documentation (Gelstan).

For RT-PCR, first strand cDNA was synthesized by mixing 11µl of total RNA with 1µl of antisense primers or OligodT. The mixture was incubated 65°C for 5 min and rapidly cooled on ice. Four µl of 5X reverse transcription buffer, 2µl of 10mM dNTPs mix, 0.5µl of RNase inhibitor and1µl of M-MuLV reverse transcriptase were added with the mixture incubated 42°C for 60 min. (Reverse Aid, First Strand cDNA synthesis kit, M/s.Thermo Fisher Scientific, USA) then the reaction mixture was incubated at 70°C for 10 min and rapidly cooled on ice. Thereafter, the cDNA was used as template for further PCR amplification

Preliminary diagnosis for AcSBV was done by subjecting the total RNA of all the samples to PCR using AcSBV primers (Grabensteiner et al., 2001). The primers sequences SBVFP (2351- 2370) 5' GTG GCA GTG TCA GAT AAT CC 3' and SBVRP (3166-3185) 5' GTC AGA GAA TGC GTA GTT CC 3' were used for detection. The PCR was performed in a thermocycler (Applied Bio systems, Thermo scientific, USA). PCR was performed with 25µl reaction volume containing 12.5µl of 2 X smART master mix, Cat. No. 280311 (readymade mix of taq polymerase, dNTPs and PCR Buffer), 5µl of cDNA, 3.5µl of sterile distilled water and 2µl of each forward and reverse primer. PCR conditions for amplification were initial denaturation at 95ºC for 5 minutes, followed by 40 cycles each consists of denaturation for 20 sec. at 95ºC, annealing for 20 sec. minutes at 55°C with final extension for 5 minutes at 72 °C followed by final elongation for 10 minutes at 72 °C.

For Reverse Transcriptase - Loop-mediated isothermal amplification, a set of specific RT-LAMP primers for AcSBV was designed based on conserved sequences in 27 isolates of AcSBV full length genome sequences retrieved from NCBI database (https://www.ncbi.nlm.nih.gov/) as given in the table 1. After retrieving, the sequences were aligned by Bio Edit software and primer set was designed using Primer Explorer version 5 software (https://primerexplorer.jp/e/). Two LAMP external primers, F3 and B3, two internal primers FIP and BIP, and the loop primer (B-loop), were designed for AcSBV (Figure 1). Desalted F3, B3, BL, FIP and BIP primers were synthesized by M/s. Precision Scientific Company Pvt Ltd. Primer sequences are shown in Table 2. cDNA was synthesized using total RNA isolated from the infected tissue.

The RT-LAMP assay was conducted in a total 25µl of reaction mixture containing 0.2µM of each outer primers (F3 and B3), 2µM of each inner primers (FIP and BIP) and 1.0 µM loop primer (Table 1), 1.6mM dNTPs, 4mM $MgSO_4$, 4 M betaine (Sigma Aldrich,

Table 1. AcSBV of 27 isolates of full length genome sequences (from NCBI database)

S. No.	Isolate	Accession Number	Nucleotide Identity (%)
1.	AcSBV-IndII2	JX270795	97
2.	AcSBV-IndK1A	JX270796	97
3.	AcSBV-IndK5B	JX270797	97
4.	AcSBV-IndK3A	JX270798	95
5.	AcSBV-IndS2	JX270799	95
6.	AcSBV-IndII9	JX270800	98
7.	AcSBV-IndII10	JX194121	96
8.	AcSBV-Kor	HQ322114	92
9.	AcSBV-VietSBM2	KC007374	94
10.	AcSBV-ChiFZ	KM495267	92
11.	AcSBV-ChiSXnor	KJ000692	93
12.	AcSBV-ChiBJ2012	KF960044	92
13.	AcSBV-ChiCQA	KC285046	90
14.	AcSBV-Kor3	KP296802	92
15.	AcSBV-Kor4	KP296803	91
16.	AcSBV-VietLD	KJ959613	96
17.	AcSBV-VietHYnor	KJ959614	91
18.	AcSBV-Viet1	KM884990	91
19.	AcSBV-Viet2	KM884991	91
20.	AcSBV-Viet3	KM884992	91
21.	AcSBV-Viet5	KM884994	91
22.	AcSBV-VietBP	KX668139	93
23.	AcSBV-VietNA	KX668140	93
24.	AcSBV-VietBG	KX668141	93
25.	AcSBV-ChiCQ1	KJ716805	90
26.	AcSBV-ChiCQB	KJ716806	91
27.	SBV-Chi	AF469603	93

St Louis, MO), 8 U of Bst polymerase (New England Biolab, Ipswich, MA), 1x Thermo Pol reaction buffer (New England Biolab, Ipswich, MA) and 2.0µl of cDNA template. The RT-LAMP reaction was carried out by incubating the reaction mix tubes at 63°C for 90 min followed by incubation at 80 °C for 10 min to inactivate the Bst polymerase enzyme. RT-LAMP amplicons were detected by adding 2µl of HNB (Hydroxy naphthol blue) and observing change in the colour of the reaction solution. Sky blue florescence could be observed clearly with naked eye in the positive reaction, where as it remained in its original dark blue in the negative reaction.

RESULTS AND DISCUSSION

RT-PCR

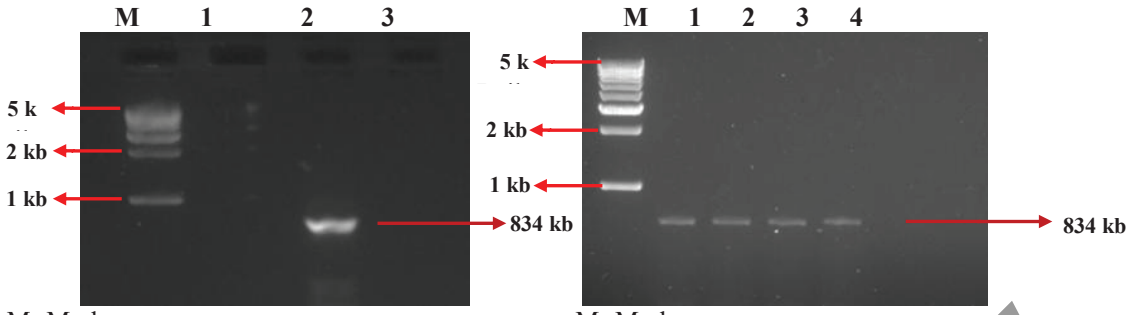
The total RNA was isolated from the SBV diseased pre pupae of *A. cerana indica* using a protocol described in materials and methods. For detection, a set of primers, SBVFP and SBVRP were used for RT-PCR amplification. This produced a fragment appoximately of 834 bp size. The amplicons were observed as clear and distinct bands under UV transilluminator. Five among ten samples were detected to have the virus. The primer pairs produced the clear and distinct bands of molecular sizes. *i.e.* approximately 834bp based on Grabensteiner et al. (2001) (Fig. 1). Gong et al. (2016) detected the sacbrood virus from *A.cerana indica* by RT-PCR method wherein, they used specific primer of sacbrood virus (SB14f and SB15r) to amplify 579bp of RNA dependent RNA polymerase (RdRp) region. Similarly reports were obtained by Freiberg et al. (2012), Rana et al. (2011), Kukielka and Sanchez-Vizcaino (2009), and Chen et al. (2004) who used specific primers to amplify the different regions of SBV at 267, 487, 597 and 210bp from Asiatic honeybee (*A.cerana indica*) and European honeybee (*A.mellifera*).

RT-LAMP

The RT-LAMP amplicons in the reaction tubes were visualized by adding the fluorescent dye (HNB - Hydroxy naphthol blue) to the reaction mixture. The colour of

Table 2. Primers used for RT-LAMP assay of AcSBV

Primer	Type	5'pos	3'pos	Primer Sequences (5' to 3')	Length
F3	Forward outer	37	57	CCTGTTGGATATGAGATGACT	21
B3	Backward outer	222	245	GTCGTAACATCTTCATTATTTTCG	24
FIP	Forward inner (F1c + F2)	114 61	138 78	ACGTATATTCTCCATTCGTCTCT GTGAATGGTTGGTGGCGAAG	43
BIP	Backward inner(B1c + B2)	143 200	165 221	GAATGTATGATGCTGAAGTGGGA ATGACTTCACTAGATACTGAGT	45
LF	Loop Forward	81	97	GCGCGTGCCATTTTGCA	17
LB	Loop Backward	166	186	TCTTTGCGTTTGGAAGACCCA	21



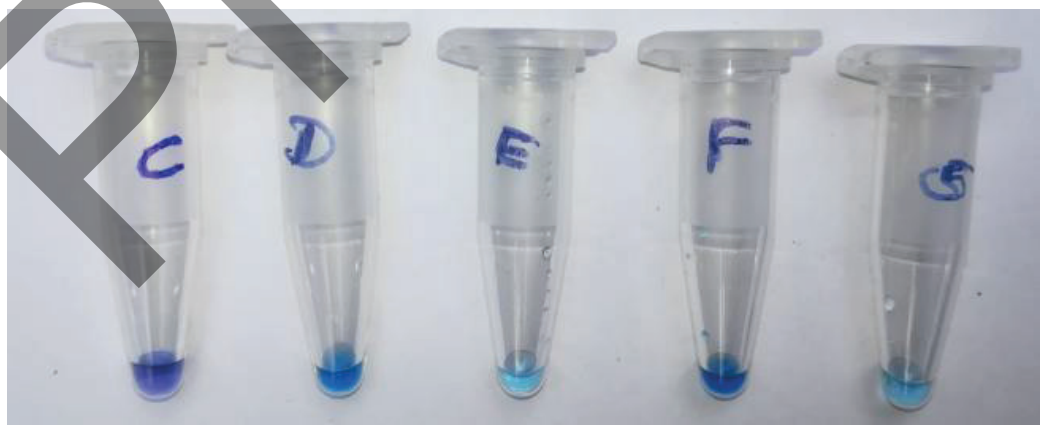
M- Marker
Lane 1 – Pollachi, Coimbatore
Lane 2 – TNAU, Coimbatore lane
Lane 3 – Sivagiri, Erode

M- Marker
Lane 1 – Periyayakanpalyam, Coimbatore
Lane 2 – AC & RI, Killikulam
Lane 3 – Marthandam, Kanyakumari
Lane 4 – Pollachi, Coimbatore

Fig. 1. Detection of sac brood virus from A.cerana indica- RT-PCR

RT-LAMP products changed from royal blue to sky blue when SBV was detected with HNB, whereas in healthy control the original royal blue colour was retained (Fig. 2). The detection rate of RT-LAMP was 4/10 (40%) in field samples. Aruna et al. (2019) compared the RT-PCR and RT-LAMP methods for detection of AcSBV in field samples and observed per cent efficiencies at 91% and 27% respectively. The current study strived to improve the rate of success of RT-LAMP in detection of the virus by concentrating on designing primer which is pivotal for accurate detection of virus. The primers were designed based on conserved regions in 27 previously reported virsuses and following step by step procedures using Primer Explorer version 5 software. Thus the detection levels were much higher (4 out of 10 samples) compared to earlier reported studies. More so, four out of 5 samples detected by RT-PCR were detected by RT-LAMP and only one sample was not detected. Another finding by Long et al. (2012) found that the RT-LAMP acquires a detection limit of 1×10^2 copies in white turbidity assay and 1×10^1 copies for analysis of SYBR Green I by RT-LAMP.

The study concluded that though slightly more time consuming, RT-PCR can be a reliable method in detecting the AcSBV with the currently designed primer sets. With respect to RT-LAMP which is more specific and depends on four primer sets targeting six regions of the genome, the chances of missing out a positive sample is more. However, in this study, a new set of primers was designed by selecting conserved regions in 27 previously reported SBV isolates so as to minimize missing out the positive samples. Thus, the existence of nucleotide level differences among the isolates was also taken into



C- Control- royal blue colour – Negative reaction; D to G – AcSBV infected samples from TNAU – sky blue colour - Positive reaction.

Fig. 2. Detection of sac brood virus from A.cerana indica- RT-LAMP

account. At the current level of research undertaken, RT-PCR is recommended as the method to diagnosing the virus with a high level of success in the laboratory while field level diagnosis can be done with RT-LAMP with reasonable level of accuracy in short time.

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