NUCLEIC ACID BASED IDENTIFICATION AND DIVERSITY STUDY OF MAJOR HONEYBEE (*Apis mellifera* L.) VIRUSES IN JAMMU REGION

By

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(J-18-M-550)

Thesis submitted to Faculty of Postgraduate Studies
in partial fulfillment of requirements
for the degree of

MASTER OF SCIENCE IN AGRICULTURE
ENTOMOLOGY

Division of Entomology
Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu
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M.Sc. (Ag.)

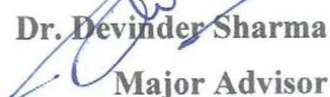
NUCLEIC ACID BASED IDENTIFICATION AND DIVERSITY STUDY OF MAJOR HONEYBEE (*Apis mellifera* L.) VIRUSES IN JAMMU REGION

Ram Narayan Sharma

2021

CERTIFICATE-I

This is to certify that the thesis entitled **"Nucleic acid based identification and diversity study of major honeybee (*Apis mellifera* L.) viruses in Jammu region"** submitted in partial fulfillment of the requirement for the degree of **Master of Science in Agriculture (Entomology)** to the Faculty of Post-Graduate Studies, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu is a record of bonafide research carried out by **Mr. Ram Narayan Sharma,** Registration No. **J-18-M-550** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that such help and assistance received during the course of thesis investigation have been duly acknowledged.



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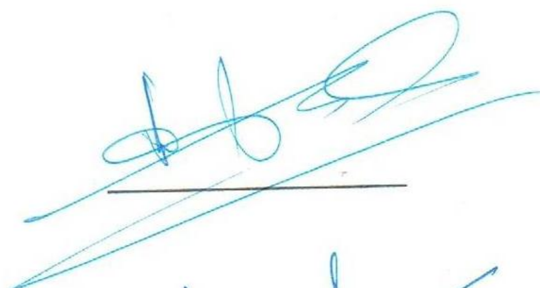

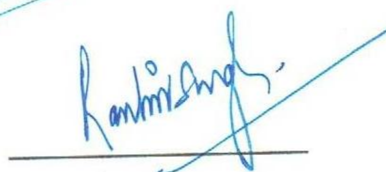

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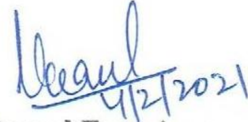

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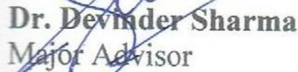

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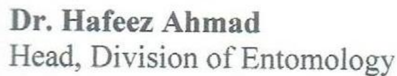

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ABSTRACT

Title of Thesis	:	Nucleic acid based identification and diversity study of major honeybee (*Apis mellifera* L.) viruses in Jammu region
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ABSTRACT

The present investigation was conducted during the year 2019-2020 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). Studies were carried on confirmation of Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), sacbrood virus (SBV) and Thai sacbrood virus (TSBV) infecting *A. mellifera* through RT-PCR technique. Specific primer for different viruses confirmed the presence of virus in selected samples of different districts, *viz.,* Jammu, Samba, Ramban and Udhampur. Results showed presence of diseased samples collected from all four districts. The primer pairs of different viruses were produced the clear and distinct bands of molecular size approximately 110, 122 and 119 bp for IAPV, KBV and SBV, respectively. The average cycle threshold (Ct) values of IAPV, KBV and SBV amplification were 26.65 ± 1.03, 17.35 ± 2.56 and 28.78 ± 4.01, respectively. Thai sacbrood virus was not detected in samples from all four districts. Israeli acute paralysis disease incidence range varied from 8.33 to 21.42 per cent. Kashmir bee disease incidence range varied from 5.71 to 28.33 per cent. Sacbrood disease incidence range varied from 8.0 to 18.75 per cent. IAPV, KBV and SBV diseases incidence range varied from 0 to 6.25, 0 to 4.16 and 0 to 9.43 per cent, respectively in the apiary at SKUAST-J, Chatha.

Keywords: *A. mellifera*, incidence, cycle threshold, IAPV, KBV, SBV, TSBV

Signature of Major advisor

Signature of Student

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LIST OF ABBREVIATIONS

bp	:	Base pair
°C	:	Degree Celsius
cDNA	:	Complementary Deoxy Ribonucleic Acid
dNTP's	:	DNA Tri-phosphates
min.	:	Minute
µg	:	Micro gram
µl	:	Micro litre
ng	:	Nano gram
%	:	Per cent
RT-PCR	:	Reverse Transcription Polymerase Chain Reaction
rpm	:	Revolution per minute
RNA	:	Ribose Nucleic Acid
TBE	:	Tris borate EDTA buffer
Uv	:	Ultraviolet
DEPC	:	Di-ethyl Pyrocarbonate
EtBr	:	Ethidium bromide
V	:	Voltage
IAPV	:	Israeli acute paralysis virus
KBV	:	Kashmir bee virus
SBV	:	Sacbrood virus
TSBV	:	Thai sacbrood virus
DWV	:	Deformed wing virus
ABPV	:	Acute bee paralysis virus
BQCV	:	Black queen cell virus
CBPV	:	Chronic bee paralysis virus
EST	:	Expressed Sequence Tag
EDTA	:	Ethylenediamine Tetraacetic Acid

Introduction

CHAPTER-1

INTRODUCTION

Honey bees occupy a prime position among all the pollinating agents because they can be managed in artificial nests, i.e. hives and can be placed in desired numbers whenever and wherever required (Goderham, 1950). Honey bees are social insects which are directly beneficial to man. They provide valuable products like pollen, royal jelly, bees wax, propolis, venom, besides providing the main product honey. Further, honey bees play vital role in agriculture by assisting in pollination of a wide variety of crops and help in maintaining biological diversity (Johannesmeier and Mostert, 2001). At present, four species of the genus *Apis* are known for honey production and pollination of crops, *viz*. *Apis cerana* F., *A. dorsata* F. and *A. florea* F. that are native to India, whereas, *A. mellifera* L. was introduced in the country during 1960 (Atwal and Goyal, 1973).

Apis mellifera (European honey bee or western honey bee) is a species of crucial economic, agricultural and environmental importance. Due to the activities of beekeepers it has now spread across the entire world, but its native range is large and diverse, spanning Europe, Africa and the Middle East (Arias and Sheppard, 2005; Han *et al*., 2012). *Apis mellifera* has been used for honey production and pollination services for thousands of years.

Recently, honeybee population experienced losses worldwide due to unknown causes. Several pathogens have been proposed to explain these losses. Some viruses appear to be the most probable cause of bee's death, such as deformed wing virus (DWV) (Martin *et al*., 2012) or acute bee paralysis virus (ABPV) (Govan *et al*., 2000). Although Israeli acute paralysis virus (IAPV) was first describe as a predictive marker of colony losses in USA (Cox-Foster *et al*., 2007), subsequent surveys indicate that this virus was not the main factor responsible for losses but only one of multiple possible factors involved (de Miranda *et al*., 2010). A possible cause of the unexplained bee losses could be combinations of pathogens such as an Invertebrate iridescent virus (IIV) with *Nosema ceranae* (Bromenshenk *et al*., 2010) or DWV with *Varroa destructor* (Dainat *et al*., 2011). Infestation with the ectoparasitic mite *Varroa destructor* is the major predisposing factor; however, a variety of other weakening circumstances may play a role in clinical manifestation of bee virus infections (e.g.,

Nosema apis infestation, intoxications, environmental pollution, and cold weather) (Suchail *et al.,* 2004).

Viruses are one of the most major threats to the health and well-being of honeybees. Viruses were first identified as a new class of pathogens infecting honeybees when at the beginning of the 20th century; a US scientist discovered that a filterable agent from diseased bee larvae could cause sacbrood disease in the honeybee. Till date 24 viruses have been reported to infect honeybees worldwide, primarily positive- strand RNA viruses belonging the families Dicistroviridae and Iflaviridae. These viruses are able to infect the different developing stages of the honeybees, including eggs, larvae, pupae and adult. Although bee viruses usually persist as unapparent infections and cause no overt signs of disease, they can dramatically affect honeybee health and shorten the lives of infected bees under certain conditions. Although usually not associated with clinical symptoms, viruses in certain cases may cause serious or lethal disease in individual bees or the collapse of entire colonies. In particular, 7 of these viruses are considered to be the cause of severe disease in honeybees threatening the world beekeeping these include acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV) and sacbrood virus (SBV).

Sacbrood disease of *A. mellifera* was noticed for the first time in USA (White, 1917). Sacbrood virus persists as in apparent, sub-lethal infections, only occasionally causing outbreaks or mortality under natural conditions (Bailey and Ball, 1991). Severity of infection in colonies is found from a few cells per comb to 90 per cent of the brood. The number of colonies infected per apiary has been found to vary from 0 to 100 per cent (Shimanuki *et al*., 1992). In India, its first incidence was reported from Kangra district of Himachal Pradesh during 1998 where it affected 2.52 to 2.92 per cent of brood (Chandel *et al*., 1999). Later, it was reported to be prevalent in other parts of Himachal Pradesh, Punjab and Haryana (Chhuneja and Singh, 2012; Rana *et al*., 2002).

Sacbrood virus infected colonies show the symptoms of partially perforated brood scattered among the capped brood, containing prepupae with typical raised head; mortality of brood in prepupal stage; sac-like appearance of dead brood; failure of prepupa to pupate; and change in colour of diseased prepupa from white to pale

yellow, to light brown and finally dark brown, which on drying turn into scales easily removable from the cells (Hornitzky and Anderson, 2003; Shen *et al.,* 2005; Berenyi *et al*., 2006).

Thai sacbrood virus (TSBV) infects larvae of the honey bee *A. cerana* resulting in failure to pupate and death of the larva. TSBV specifically infects *A. cerana* and is not known to cause infection on *A. mellifera* (Aruna *et al*., 2016).

Israeli acute paralysis virus (IAPV) was first described in 2004 in Israel, where severe bee mortality caused heavy losses to Israeli apiculture (Maori *et al*., 2007). IAPV was characterized as a new member of the Dicistroviridae family (Christian *et al*., 2005), closely related to Kashmir bee virus (KBV) and Acute bee paralysis virus (ABPV), but genetically and serologically distinct (Maori *et al.* 2007). Recently, the presence of IAPV has been strongly correlated with a new syndrome of honey bee losses observed in the United States, called Colony Collapse Disorder (CCD) (Cox-Foster *et al*., 2007).

Diagnosis of viral diseases in the field is based on visual inspection of brood-combs and detection of diseased larvae. However, symptoms of viral diseases may be easily confused with other diseases or abnormalities in the brood making definitive diagnosis difficult. Hence, confirmatory laboratory assays, such as ELISA and PCR (Polymerase chain reaction) are used to verify the presence of sacbrood virus in suspected colonies. Detection of honey bee viruses has been done through several methods including immune gel diffusion technique (Allen and Ball, 1996), electron microscopy (Rana and Rana, 2015), enhanced chemiluminescent western blotting (Allen *et al*., 1986) and reverse transcriptase (Rana *et al*., 2011). Research is also being conducted on the characterization of honey bee viruses on molecular basis through RT-PCR technique in different parts of the world (Grabensteiner *et al.,* 2001; Rana *et al.,* 2011).

Jammu and Kashmir is one of the most important beekeeping areas in India. At least four agroclimatic zones ranging from low altitude subtropical, intermediate, temperate and cold alpine occur. Temperatures range from –45°C to 45°C and above. Such diversity of geographical features plays a dominant role in determining the topography, climate and plant species present in the region. It offers great potential for both migratory and non-migratory beekeeping.

Presence of various diseases, predators and pests which impair the health and normal working of *A. mellifera* is one of the major problems among beekeepers in Jammu and Kashmir. The knowledge about the seasonal incidence of various diseases and enemies of *A. mellifera* under changing scenario is scarce and there is need for qualitative survey of pests and pathogens of *A. mellifera* under different conditions. Further, the prospective beekeepers should be made aware of various limiting factors to protect honey bee colonies to get maximum benefit from beekeeping venture. To check the prevalence of different viral disease present in the region, the present study on *A. mellifera* was, therefore, undertaken with the following objectives:

1. To develop a nucleic acid based method for identification of major viral diseases of honey bee.
2. To survey the incidence of major honeybee viral diseases in the Jammu region.

Chapter-2

Review of Literature

CHAPTER-2

REVIEW OF LITERATURE

Honey bees are very important social and economically valuable insects due to their honey production and pollinating activities (Lawal and Banjo, 2010). Honey bee produces honey, bee wax, royal jelly, pollen, propolis and bee venom. Honey is one of the most nutritive foods containing various kinds of sugars, proteins, free amino acid, mineral, trace element, enzymes and vitamins with a fairly high caloric value.

Honey bee colony is a rich source of food with brood, adults, honey, pollen and beeswax. They are attacked by various pests, predators and other enemies. In India, virus diseases, such as acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), sacbrood virus (SBV) and thai sacbrood virus(TSBV) have been reported in hive bees. Absconding, swarming and drifting behavioural aspects help the disease to spread with greater ease within the colony, colony to colony and apiary to apiary (Bailey, 1963).

The available literature on *A. mellifera* virus diseases is presented under following headings:

2.1 Nucleic acid based method for identification of major viral diseases of honey bee

Bailey and Woods (1977) isolated black queen cell virus from prepupae and pupae of queens and workers of *Apis mellifera*. Kashmir bee virus was isolated from individuals of *Apis mellifera* that had died in the laboratory after they had been inoculated with some preparations from *Apis cerana*.

Bailey *et al.* (1982) isolated a strain of sacbrood virus from larvae of the Eastern honey bee, *Apis cerana* from Thailand. This strain of virus, which they called Thai sacbrood virus (TSBV), had physical as well as serological properties that distinguish it from the type strain of sacbrood virus (SBV), which came from the European honey bee, *Apis mellifera*.

Ghosh *et al.* (1999) determined the nucleotide sequence of sacbrood virus (SBV), which causes a fatal infection of honey bee larvae. The genomic RNA of SBV was longer than that of typical mammalian picornaviruses (8832 nucleotides) and contained a single large open reading frame (179-8752) encoding a polyprotein of

2858 amino acids. Sequence comparison with other virus polyproteins revealed regions of similarity to characterized helicase, protease and RNA-dependent RNA polymerase domains; structural genes were located at the 5' terminus with non-structural genes at the 3' end.

Benjeddou *et al.* (2001) developed a reverse transcriptase PCR (RT-PCR) assay for the detection of acute bee paralysis virus (ABPV) and black queen cell virus (BQCV). Complete genome sequences were used to design unique PCR primers within a 1-kb region from the 3′ end of both genomes to amplify a fragment of 900 bp from ABPV and 700 bp from BQCV. The combined guanidinium thiocyanate and silica membrane method was used to extract total RNA from samples of healthy and laboratory-infected bee pupae. They concluded that in a blind test, RT-PCR successfully identified the samples containing ABPV and BQCV. Sensitivities were approximately 1,600 genome equivalents of purified ABPV and 130 genome equivalents of BQCV.

Evans (2001) used nucleotide sequence analyses to identify acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) isolated from a single honey bee colony. He stated that most of the bees in the colony carried KBV, while some individual bees also carried ABPV, a coexistence not yet seen between these two viruses.

Grabensteiner *et al*. (2001) reported failure of sacbrood virus infected larvae of *A. mellifera* to pupate. Identification of viruses in honey bee was done through electron microscopy, immunodiffusion and enzyme-linked immunosorbent assay but as the methods were low in sensitivity and specificity, a reverse transcription (RT-PCR) test for the direct, rapid and sensitive detection of these viruses was reported.

Bakonyi *et al.* (2002) established RT-PCR assays for a quick, sensitive and specific diagnosis of acute bee paralysis virus (ABPV). A 3,071-nucleotide fragment of the ABPV genome, which included the entire capsid polyprotein gene, was amplified from Austrian, German, Polish, and Hungarian ABPV samples.

Chen *et al.* (2004) screened honey bee colonies using uniplex RT-PCR for the presence of several bee viruses [black queen cell virus (BQCV), deformed wing virus (DWV), Kashmir bee virus (KBV), and sacbrood virus (SBV)] and described the detection of mixed virus infections in bees from these colonies. Their findings revealed that out of fifty-six colonies examined, 75 per cent (42/56) colonies were

found to be infected with one or more than one virus although tested bees rarely had overt symptoms of infection. Of colonies with virus infection(s), 38 per cent (16/42) colonies had mono-infection of BQCV, DWV, KBV, or SBV, 50 per cent (21/42) colonies had dual-infection, 7 per cent (3/42) colonies had triple-infection, and 5 per cent (2/42) colonies had tetra-infection. Emphasizing that the combination of DWV and BQCV infections was observed with the highest frequency (43%, 18/42) among colonies with virus infections.

Chen *et al.* (2005) by using RT-PCR and Southern hybridization detected deformed wing virus (DWV) in all life stages of honeybees, including adults with and without deformed wings. In samples collected by them from two colonies with apparently DWV-infected worker bees, the percentages of DWV positive samples were 100 per cent for adult worker bees with wing deformities, 95 per cent for pupae, 92 per cent for parasitic mites, 80 per cent for larvae, 79 per cent for normal-looking adult bees, and 47 per cent for adult drones.

Topley *et al*. (2005) reported a single multiplex reverse transcriptase polymerase chain reaction (RT-PCR) assay for the simultaneous detection of three honey bee viruses *i.e*. acute bee paralysis virus (ABPV), sacbrood virus (SBV) and black queen cell virus (BQCV). Unique PCR primers were designed from the complete genome sequence to amplify fragments of 900 bp from ABPV, 434 bp from SBV and 316 bp from BQCV. Individual bee pupae homogenates and total RNA extracted from these crude extracts was used in the RT-PCR amplification. Furthermore, field samples of honeybee pupae were screened for viral infections and evidence of virus in apparent infection as well as virus co-infection were found.

Yue and Genersch (2005) used RT-PCR protocol for the detection of DWV, individual bees and mites originating from hives differing in *Varroa*-infestation levels and the occurrence of crippled bees were analysed. They were found that 100% of both, crippled and asymptomatic bees were positive for DWV.

Berenyi *et al.* (2006) used RT-PCR for detection of 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). They concluded that the most prevalent virus was DWV, present in 91 per cent of samples and was followed by ABPV, SBV and BQCV (68%, 49%, and 30%,

respectively). Emphasizing that CBPV was detected in 10 per cent of colonies, while KBV was not present in any sample.

Ward *et al.* (2007) developed a real-time PCR assay for specific detection of Kashmir bee virus (KBV). KBV was successfully amplified from different life stages of honey bees, wasp and bumble bee. KBV was detected within three colonies at two locations. The virus titre in the positive samples was quantified and found to contain similar levels to other bees with covert KBV infection. They concluded that KBV is present in the UK and cannot now be considered an exotic disease.

Baker and Schroedar (2008) reported that single-stranded RNA viruses infectious to the European honey bee, *A. mellifera* were present at low levels in colonies, with typically no apparent signs of infection observed in the honey bees. Reverse transcription-PCR (RT-PCR) of regions of the RNA-dependent RNA polymerase (RdRp) is often used to diagnose their presence in apiaries and also to classify the type of virus detected.

Teixeira *et al.* (2008) developed and validated a multiplex RT-PCR assay for the simultaneous detection of ABPV, BQCV, and DWV in Brazil. Three different bee viruses, *viz a viz.,* acute bee paralysis virus (ABPV), black queen cell virus (BQCV) and deformed wing virus (DWV) which were identified during the screening of RNAs from 1920 individual adult bees collected in a region of southeastern Brazil that has recently shown unusual bee declines. They emphasized that ABPV was detected in 27.1 per cent of colony samples, while BQCV and DWV were found in 37 per cent and 20.3 per cent, respectively.

Thi *et al.* (2008) developed a PCR method to detect and verify sacbrood virus (SBV), which affects the honeybee larvae. The PCR assay was performed with SBV specific primer set and amplified 824 bp fragments from SBV genome. SBV3- 842F/R primer set showed high specificity and sensitivity in detection of SBV. SBV amplicon was cloned and sequenced to make sure result of PCR and digestion.

Kukielka and Vizcaino (2009) conducted two one-step real-time RT-PCR assays for the detection, differentiation and quantitation of two important honey bee viruses: Sacbrood virus (SBV) and acute bee paralysis virus (ABPV). Both reactions were optimized to yield the highest sensitivity and specificity. Viral detection and identification were confirmed by melting curve analysis and sequencing of the PCR

products. Both techniques were used to evaluate Spanish field samples and establish the distribution of these viruses. Acute bee paralysis virus was not detected and sacbrood virus was present at low frequencies. The one step real-time RT-PCR methods are fast, accurate, and useful for detecting and quantifying these honey bee viruses, which cause in apparent infections and contribute to the increasing depopulation of honey bee colonies.

Sanpa and Chantawannakul (2009) conducted survey on six honey bee viruses using RT-PCR in Northern Thailand where about 80 per cent of Thai apiaries are located. Tested samples were found to be positive for deformed wing virus (DWV), acute bee paralysis virus (ABPV), sacbrood virus (SBV) and Kashmir bee virus (KBV).

Yan *et al*. (2009) screened by the RT-PCR method for the presence of 6 honey bee viruses, including the Israeli acute paralysis virus (IAPV). Two viruses were detected, including deformed wing virus (DWV) from all dead and live workers and IAPV from dead workers only in one of 4 sampling locations. None of the six viruses were detected from the broods. The sequence and phylogenetic status of the detected IAPV were analyzed. This is the first detection of IAPV in colonies of *A. mellifera* in China.

Kajobe *et al.* (2010) used RT-PCR to screen immature and adult honey bees (*Apis mellifera* L.) which were collected from 63 beekeeping sites across Uganda for seven viral pathogens. As per their findings, no samples tested positive for chronic bee paralysis virus, sacbrood virus, deformed wing virus, acute bee paralysis virus, *Apis iridescent* virus and Israeli acute paralysis virus. However, black queen cell virus (BQCV) was found in 35.6 per cent of samples. It occurred in adults and larvae, and was most prevalent in the Western highlands, accounting for over 40 per cent of positive results nationally.

No *et al.* (2010) developed a new semi-nested PCR method to detect Israeli acute paralysis virus (IAPV) which is the causative virus of colony collapse disorder (CCD) in honeybee. They concluded that the unique 284 bp DNA fragment was successfully amplified with high sensitivity using IAPV specific semi-nested PCR method. From only three copies of IAPV-template, the IAPV specific semi-nested PCR showed positive amplification. From field sample its sensitivity was also verified.

They added that the IAPV semi-nested PCR method would be applicable to the early detection of IAPV and the confirmation test for existence of few IAPVs in nature.

Rana *et al.* (2011) detected sacbrood virus through ELISA and RT-PCR techniques in diseased prepupae of *A. mellifera* collected from different apiaries of Himachal Pradesh, India. The ELISA kit was prepared from antiserum specific to Thai sacbrood virus. Sacbrood virus in the crude samples was detected at 1:1000 titre of the kit. Five pairs of oligonucleotide primers were used to target sacbrood virus genome through RT-PCR technique. Presence of sacbrood virus was confirmed with 3 primers.

Francis and Kryger (2012) used RT-PCR technique for identifying acute bee paralysis virus (ABPV), Kashmir bee virus (KBV) and Israeli acute bee Paralysis virus (IAPV) of honey bees (*Apis mellifera* L.). The study recorded the first case of IAPV in Denmark. Further, out of 87 samples, 46 samples were found to be positive for ABPV, 6 for KBV and 6 for IAPV.

Gregorc and Bakonyi (2012) reported that viral infection could have an impact on the success of queen rearing and a potential effect on reduced queen quality. Newly mated honey bee (*A. mellifera carnica*) queens were collected from mating nuclei in queen rearing operations in Slovenia. Queens were analyzed for the presence of four viruses: acute bee paralysis virus (ABPV), black queen cell virus (BQCV), sacbrood virus (SBV) and deformed wing virus (DWV) by using reverse transcription polymerase chain reaction (RT-PCR). In 2006, 12 per cent, 9 per cent and 1 per cent prevalence was found for ABPV, DWV and SBV, respectively; BQCV was not detected. Two years later, DWV, BQCV, SBV and ABPV were detected in 58 per cent, 24 per cent, 11 per cent and 10 per cent bee queens, respectively. In 2006, 14 out of 27 apiaries were virus free, whereas in 2008 only 3 out of 24 apiaries were virus free. This is the first evidence of virus infection occurring in newly mated queens from mating nuclei in rearing apiaries.

Yoo *et al.* (2012) developed a real-time reverse transcription-polymerase chain reaction (qRT-PCR) assay for the fast and highly sensitive detection of the sacbrood virus (SBV) genome and applied to honey bee samples. Aimed to reduce the detection time for SBV, synthesis of the cDNA of the SBV genome from a honey bee sample was attempted for different reaction times and the cDNA was used as the template for URRT-PCR assays. The results indicated that a 5 min reaction time was sufficient to

synthesize cDNA as the template for the SBV URRT-PCR assay. This study described a novel PCR-based method that is able to detect an RNA virus in environmental samples within 22 min, including reverse transcription, PCR detection and melting point analysis in real-time.

Sguazza *et al.* (2013) established a multiplex polymerase chain reaction method for rapid and simultaneous detection of the most prevalent bee viruses. They found that multiplex PCR assay provided specific, rapid and reliable results and allowed for the cost effective detection of a particular virus as well as multiple virus infections in a single reaction tube. No differences were observed between the mRT-PCR described in this work and other simplex PCRs previously reported. Even more, they did not find differences between simplex or multiplex PCR in the 170 samples analyzed by both methods. They have found a frequency of 45.9 per cent for IAPV, 37.1 per cent for DWV, 12.3 per cent for CBPV, 13, 6 per cent for SBV, 3.5 per cent for BQCV and 7.7 per cent for ABPV. Finally, 29 samples were found positive to different combinations of two viruses; most of them (17 samples out of 29) had the association of IAPV and DWV.

Garcia-Anaya *et al.* (2018) used RT-PCR technique to detect two major viral diseases *viz*, *Apis mellifera* filamentous virus (AmFV) and Israeli acute paralysis virus (IAPV) in honey bees in Mexico. Adult and larvae honey bee samples were collected from asymptomatic colonies of six major beekeeping regions in the state of Chihuahua, Mexico. Both viruses were detected in both developmental stages of honey bees. IAPV at a higher prevalence (23.5%) as compared to AmFV, only in 0.9 per cent of samples. However, this is the first report on AmFV infection in Mexican apiaries.

Cagirgan and Yazici (2020) developed a multiplex RT-PCR (mRT-PCR) test to obtain faster results in routine diagnostic laboratories for seven crucial bee viruses. Specific primers for seven RNA viruses, including Israeli acute bee paralysis virus (IAPV), deformed wing virus (DWV), sacbrood virus (SBV), acute bee paralysis virus (ABPV), black queen cell virus (BQCV), Kashmir bee virus (KBV) and chronic paralysis virus (CBPV), were used for testing procedure. They mentioned that the mRTPCR assay could amplify seven plasmid DNA fragments from the pooled viral genomes and it was shown to be sensitive because virus copy numbers were detected

to be 10^4 copies/µl when $\log_{10}$ serial dilutions were performed for the optimized mRT-PCR method.

2.2 Incidence of major honeybee viral diseases

Anderson (1990) on a survey to study the occurrence and distribution of pests and pathogens of the honey bee, *A. mellifera* in Fiji, South Pacific. He found that American foulbrood disease was detected in 33 (47%) hived colonies at one locality and in only one (0·8%) of all other colonies examined in other localities. Also, added that chronic bee paralysis virus (CBPV) and sacbrood virus (SBV) were present in 30 (31%) and 76 (79%) hived colonies, respectively. Bee virus X, bee virus Y, black queen cell virus (BQCV), Kashmir bee virus (KBV) and slow bee paralysis virus were also detected in these colonies but they were less common than CBPV and SBV. In apparent infections of SBV, BQCV and KBV were detected in seemingly healthy bees from hived colonies. SBV and CBPV were also detected in feral colonies. *Nosema apis* was found in 45 (47%) hived colonies.

Bowen-Walker *et al.* (1999) observed that under field conditions, *Varroa jacobsoni* were shown to be highly effective vectors of deformed wing virus (DWV) between bees. Adult female mites obtained from honeybee pupae naturally infected with DWV contained virus titers many times in excess of those found in their hosts and, beyond that, which could have been expected from a concentration effect. Therefore, it may be possible that DWV may be capable of replicating with in *V. jacobsoni.* Bees which tested positive for DWV exhibited characteristic morphological deformity and/or they died during pupation. Mites (462) in three trials were introduced into a total of 179 recently capped worker cells. Of these, 97 bees (54%) survived to emergence.

Tentcheva *et al*. (2004) detected six bee viruses in *A. mellifera* colonies in France by using the PCR technique. In adult bees, deformed wing virus (DWV), sacbrood virus (SBV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), black queen cell virus (BQCV) and Kashmir bee virus (KBV) was 97 per cent, 86 per cent, 28 per cent, 86 per cent and 17 per cent, respectively. In addition, the frequencies of viruses in pupae of *A. mellifera* apiaries were 94 per cent, 80 per cent, 23 per cent, 23 per cent and 6 per cent for DWV, SBV, ABPV, BQCV, KBV and none for CBPV.

Rana and Rana (2005) detected sacbrood virus at two locations in Himachal Pradesh. At Nauni (district Solan), it was detected in colonies during spring and summer (March- May) affecting 0.39 per cent to 5.20 per cent of the brood while at Jachh (district Kangra), the disease was also detected during March to June infecting 0.23 per cent to 2.10 per cent of brood.

Berenyi *et al.* (2006) observed that sacbrood virus and Thai sacbrood virus infected colonies of *A. mellifera* and showed the symptoms of partially perforated brood, scattered among the capped brood, containing prepupae with typical raised head, mortality of brood in prepupal stage, sac-like appearance of dead brood, failure of prepupa to pupate, change in colour of diseased prepupa from white to pale yellow to light brown and finally dark brown which on drying turn into scale which are easily removable from the cells. Sacbrood disease is caused by the sacbrood virus which affects worker bee. The infected larvae die shortly after capping and become a fluid filled sac. Infected broods are found scattered amongst healthy brood and the capping may be discoloured, sunken or perforated. Sacbrood virus may remain viable in dead larvae, honey or pollen for up to four weeks.

Todd *et al.* (2007) studied on the virus status of honey bee colonies infested with *Varroa destructor*. They observed viruses *viz*., CBPV, BQCV, SBV, DWV, and KBV during the study, which DWV and KBV were the most common, as inferred from serological and protein profile analyses. Emphasizing that DWV, SBV and ABPV were not detected in these colonies.

Nielsen *et al.* (2008) observed the presence of 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) by RT-PCR. All six viruses were detected, but the frequencies varied significantly: emphasizing that SBV was detected in 78 apiaries, DWV in 55 apiaries, ABPV in 11 apiaries, CBPV in 4 apiaries, BQCV in 1 and KBV in 1 apiary.

Palacios *et al.* (2008) found that Israeli acute paralysis virus (IAPV) is associated with colony collapse disorder of honey bees. Nonetheless, its role in the pathogenesis of the disorder and its geographic distribution are unclear. The phylogenetic analysis of IAPV obtained from bees in the United States, Canada, Australia, and Israel and the establishment of diagnostic real-time PCR assays for

IAPV detection indicated the existence of at least three distinct IAPV lineages, two of them circulating in the United States.

Rana and Rana (2008) reported that sacbrood virus killed honey bee brood at the prepupal stage (10 days of age) on the second day after the sealing of brood. Sacbrood virus was detected in colonies during spring and summer (March to May) affecting 0.39 per cent to 5.20 per cent of the brood.

Ai *et al.* (2012) surveyed on the populations of *Apis mellifera* and *Apis cerana* for seven bee viruses *viz a viz* acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Kashmir bee virus (KBV), sacbrood virus (SBV) and Isreali acute paralysis virus (IAPV). They reported that in *A. mellifera*, DWV was the most prevalent virus, but in *A. cerana*, SBV was the dominant prevalent virus. Emphasizing simultaneous multiple infections of viruses were common in both species. In *A. mellifera*, DWV was found to be the most prevalent, present from 94 per cent of the apiaries. BQCV, SBV, IAPV, ABPV and CBPV were present in 44 per cent, 21 per cent, 18 per cent, 6 per cent and 9 per cent of the apiaries, respectively. The virus prevalence from the apiaries of *A. cerana* was quite different. SBV was the dominant and present in 86 per cent of the apiaries. This was followed by DWV present in 64 per cent of the apiaries, and IAPV, CBPV or BQCV present in 7 per cent of the apiaries, respectively. There was no KBV reported from any samples of the two species.

Freiberg *et al.* (2012) in a study witnessed sacbrood of *A. mellifera* characterized by the brood that failed to pupate and subsequently died, the decline was reported as an important threat to honey bee health.

Dar and Kotwal (2014) conducted a survey for the detection of deformed wing virus (DWV) using RT-PCR and specific primers and found that deformed wing virus (DWV) was found in 80 per cent of the apiaries and infection was increased from spring to summer during the year both in adults and pupae, but decreased in autumn as colony brood declined. Mainly emphasizing on the recorded DWV frequency in the spring and summer was 37 and 65 per cent respectively, but in autumn, it was 32 per cent. With respect to pupae, it was 16, 26 and 7 per cent respectively. As a whole, higher virus frequency was detected in brood populations (92%) than adult populations (90%).

Gajger *et al.* (2014) reported that viral honey bee diseases are an increasing beekeeping problem throughout the world due to great economic losses and a reduction in biodiversity in natural ecosystems. They also added the viruses identified were deformed wing virus (DWV) with 100 per cent, sacbrood bee virus (SBV) with 70 per cent, black queen virus (BQCV) with 20 per cent, acute bee paralysis virus (ABPV) with 10 per cent and chronic bee paralysis virus (CBPV) with 10 per cent incidence. Multiple infections of the examined honey bee colonies were found in 80 per cent of samples.

Predrag *et al.* (2014) conducted a survey on 55 honey bee colonies which were monitored for the presence of deformed wing virus (DWV) and acute bee paralysis virus (ABPV) using TaqMan-based real-time RT-PCR assay. They observed the high frequency of DWV (76.4%) and ABPV (61.8%). DWV was found to be more prevalent than ABPV.

Rana and Rana (2015) reported that the presence of sacbrood virus (SBV) in colonies of the western honey bee (*A. mellifera*). The virus killed honey bee brood at the prepupal stage (10 days of age) on the second day after the sealing of brood. SBV was detected at two locations in Himachal Pradesh, Solan district, Nauni, it was detected in colonies during spring and summer (March to May) when it affected 0.39 per cent to 5.20 per cent of the brood. At the second location, Kangra district, Jachh (Himachal Pradesh), the disease was also detected during spring and summer (March to June) when it affected 0.23 per cent to 2.1 per cent of brood. The incidences of the disease were found to be significantly correlated with colony strength and brood rearing.

Gong *et al.* (2016) found that the sacbrood virus is a single-stranded, positive sense RNA virus belonging to the genus Iflavirus in the family Iflaviridae. The virus primarily affects the brood stage of honey bees causing failure of pupation and larval death but, also affects adult bees without obvious signs of the disease. Sacbrood is characterized by the diseased infected larva, encased in a tough, fluid filled sac formed from the larval cuticle.

Reddy *et al.* (2017) found that sacbrood virus (SBV) is one of the most common viral infections of honeybees. They collected the samples from Vietnam and sequences were aligned with seven previously reported complete genome sequences of

SBV from other countries, and various genomic regions were compared. The samples shared 91-99 per cent identity with each other, and shared 89-94 per cent identity with strains from other countries. Studies indicate that host specificity, geographic distance, and viral cross infections between different bee species may explain the genetic diversity among the SBV in *A. cerana* and *A. mellifera* and other SBV strains.

Karapinar *et al*. (2018) determined the presence and prevalence of viral and parasitic infections. The samples were tested by Reverse-Transcriptase PCR (RT-PCR) for acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV) and deformed wing virus (DWV). Selected positives were sequenced, phylogenetically analyzed and investigated in terms of *Varroa*. DWV and BQCV were identified in 69.23 per cent (18/26) and 88.46 per cent (23/26) of the bees respectively whereas ABPV and CBPV were not detected in the sampled apiaries.

Rustemoglu *et al.* (2019) conducted a survey on the occurrence and prevalence of black queen cell virus (BQCV), deformed wing virus (DWV), sacbrood virus (SBV), chronic bee paralysis virus (CBPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV). The presence of BQCV, DWV, SBV and CBPV infections were ascertained for the first time by RT-PCR. BQCV was the most frequently detected virus, found in 32 per cent of bee samples, less than 24 per cent of asymptomatic bee samples were positive for DWV, SBV and CBPV with the infection ratio of 23, 12 and 9 per cent, respectively. During this survey, KBV and IAPV were not detected in any of tested bee samples. Some double and triple infections were encountered: 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%).

Chapter-3

Materials and Methods

CHAPTER-3

MATERIALS AND METHODS

The present investigation was carried out at Stress Biology Laboratory of Division of Livestock Production and Management, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-J, R. S. Pura, Jammu, during the year 2019-2020. The research material used and methodology followed to conduct this study has been described under the following headings:

3.1 Reagents and Solutions

3.1.1 Reagents

(a) Primers

(b) dNTPs: (dATPs/ dCTPs/ dGTPs/ dTTPs) 100 mM stock of each dNTP was diluted to 2.5 mM of dNTP.

(c) PCR buffer 10 X

(d) Chloroform

(e) Isopropanol

(f) Ethanol

3.1.2 Stock solutions

Different reagents and buffers used for RNA extraction, gel electrophoresis and gel staining are given below:

(i) 0.5M EDTA: It was prepared by adding 186.1 g of EDTA to 800 ml of Millipore water. The pH was adjusted to 8.0 by adding NaOH pellets. Note that EDTA would not be completely dissolved until the pH is adjusted to 8.0. The final volume was raised to 1 L and autoclaved.

(ii) 1M Tris-Cl: It was prepared by dissolving 121.1 g of Tris base in 700 ml of Millipore water. The pH was adjusted to 8.0 by adding using concentrated HCl and final volume was raised to 1 L and autoclaved.

(iii) 10X TBE buffer: It was prepared by dissolving 108 g of Tris base, 55g of boric acid in 700 ml of Millipore water. Also 40 ml of 0.5M EDTA was added to this, stirred, dissolved and final volume was raised to 1 L.

(iv) 10X TE buffer: It was prepared by mixing 100 ml of Tris-Cl (Ph 7.5) and 20 ml of 0.5M EDTA and final volume was made exactly up to 1 L.

(v) Ethidium bromide solution (10mg/ml): It was prepared by dissolving 10 mg ethidium bromide in 10 ml double distilled water and stored at 4°C in dark

(vi) DEPC water: It was prepared by dissolving 1 ml DEPC in 1 L on the magnetic stirrer for overnight stirring and autoclaved.

3.2 Molecular detection through Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique

3.2.1 Sampling

Samples of virus infected *A. mellifera* larvae and adults were collected from private beekeepers apiaries of the four selected districts (Jammu, Samba, Ramban and Udhampur) as well as SKUAST-J University apiary (Plate 1). A total of 200 samples were collected with 50 samples from each district (Plate 2). Well aerated plastic vial was used for collection of samples. The vials were placed in ice box in order to preserve RNA integrity and brought to the laboratory for further PCR studies.

3.2.2 Isolation of total RNA from honey bee samples

For RNA isolation, all the glassware and plasticware were pre-treated with di-ethyl pyrocarbonate (DEPC) water. RNA was isolated from honey bee samples collected at different interval by Trizol method. The samples were crushed in motar and pestle by using liquid nitrogen. The crushed fine product was mixed with 500 µl of Tri-Reagent (Sigma-aldrich). To the 500 µl trizol containing lysed cells, 200 µl chloroform was added and vortexed briefly for uniform mixing. The suspension was centrifuged (13000 rpm for 10 min.). 200-300 µl of the supernatent was carefully aspirated and transferred to a micro centrifuge tube. 0.8 volume of Pre-Chilled isopropanol was added to this and the mixture was transferred to a RNA binding column (Zymo Research, CA, USA). The column with collection tube was centrifuged at 10000 rpm for 5 minutes. The silica pad

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

Plate 2: Survey and collection of samples from colonies

of the column was washed twice with chilled 80 per cent ethanol and dried by centrifugation at 13000 rpm for 2 minutes. The RNA bound to silica pad was eluted by adding 15 µl of DEPC treated water, followed by centrifugation (14000 rpm for 3 minutes). The RNA was stored at -20°C until further analysis.

3.2.3 Agarose gel electrophoresis

The RNA from all the honey bee sample was quantified by loading 5 µl of RNA mixed with 3 µl of loading dye into separate wells on 1.5 per cent agarose gel. 1.5 per cent agarose gel was prepared by dissolving 1.5 g of agarose in 100 ml of 1X TBE (DEPC treated Tris Borate EDTA) buffer. The agarose was melted by boiling it in microwave for 2 minutes until it became transparent. It was then allowed to cool and stained with ethidium bromide (EtBr). Ethidium bromide is an intercalating dye and intercalates between the grooves of RNA. The gel was then poured in gel casting tray with combs in it and allowed to solidify for 20-25 minutes at room temperature. The electrophoresis was carried out at 100 V for half an hour. The gel was then viewed under UV transilluminator.

3.2.4 cDNA synthesis

The cDNA was synthesized by using Hi-cDNA Synthesis kit (HI MEDIA) by following the manufacturer's protocols.

- A tube was taken and the following reagents were added (Table 1).

Table 1: Ingredients used for cDNA synthesis

Ingredients	**Quantity (µl)** **Volume per reaction**
Random Hexamer or Oligo (dT)	1 µl
RNA template	5 ng or 5 µg
Molecular Biology Grade Water for PCR	Up to 10 µl
Total	**10 µl**

- It was incubated for 5 minutes at 65ºC, then cooled immediately on ice.
- The following reverse transcription reagents were then added (Table 2).

Table 2: Ingredients used for cDNA synthesis

Ingredients	**Quantity (µl)** **Volume per reaction**
Template RNA Primer Mixture (from upper step)	10 µl
RT Buffer for MMuLV	4 µl
10X Solution for MMuLV	2 µl
M-MuLV Reverse Transcriptase (RNase H-)	1 µl
10mM dNTP mix	2 µl
Molecular Biology Grade Water for PCR	1 µl
Total	**20 µl**

- It was gently mixed and centrifuged.
- It was incubated as followed (Table 3).

Table 3: Thermal cycling parameters for cDNA synthesis

Random Hexamer	**No. of cycles**
25ºC for 5 minutes	1 cycle
42ºC for 60 minutes	1 cycle
70ºC for 5 minutes	1 cycle
Hold at 4ºC	Optional

OR

Oligo (dT)	**No. of cycles**
42ºC for 60 minutes	1 cycle
70ºC for 5 minutes	1 cycle
Hold at 4ºC	Optional

- These tubes were then stored at -20ºC or processed.

3.2.5 Primer designing

The primers were designed from available expressed sequence tag (ESTs) or reference sequence available in NCBI/EMBL database. The details of primers used for real time amplification are provided in Table 4 and 5.

Primer set with their sequence selected for amplification of cDNA

Table 4: Primer set for KBV and IAPV

Primer Name	Primer Sequence (5'- 3')	Length (bp)	Tm (^oC)
KBV3CPRTFW	CAAACTGCTGAATCAATGTCAAAAT	25	50
KBV3CPRTRV	ACATGCCTCTACTTTGTCACATTCA	25	50
IAPVVP3RTFW	CGAACTTGGTGACTTGAAGG	20	50
IAPVVP3RTRV	GCATCAGTCGTCTTCCAGGT	20	50

Table 5: Primer set for SBV and TSBV

Primer Name	Primer Sequence (5'- 3')	Length (bp)	Tm (^oC)
TSBVRTFW	AGGGAAATTACTAATATACCTTGCCTTCATATA	33	57
TSBVRTRV	GGCAACTATGGCACTCTCACCATAGTT	27	60
SACP1RTFW	GGACTCTTATACCGATTTGTTTAATGGTTGGG	32	61
SACP1RTRV	CGCGTCTAACATTCCAGATTCTTCGTCC	28	61

3.2.6 Molecular detection through reverse transcription polymerase chain reaction (RT- PCR) technique

All Real-Time PCR (RT-PCR) reactions were performed on a CFX Connect Real-Time thermal cycler machine (Bio-Rad Laboratories, Inc., CA, USA). Each reaction consisted of 2 µl cDNA template, 5 µl of 2× SYBR Green PCR Master Mix, 0.25 µl each of forward and reverse primers (10 pmol/µl) and nuclease free water for a final volume of 10 µl (Table 6). Each sample was run in duplicate.

Table 6: Reagents used for single RT-PCR reaction

S.No	**Reagents**	**Quantity**
1.	cDNA	2 µl
2.	2× SYBR Green PCR Master Mix	5 µl
3.	Primer forward	0.25 µl
4.	Primer reverse	0.25 µl
5.	Sterile water	2.5 µl
	Total	**10 µl**

Table 7: Thermal profile of RT-PCR

Steps	Cycles	Temperature(°C)	Duration
Initial Denaturation	1	95	3 min
Denaturation	35	95	10 seconds
Annealing/extension and plate read		55	30 seconds
Melt curve analysis		65-95	0.5 °C increment at 5 seconds/step
Holding		4	∞

3.2.7 Agarose gel electrophoresis of RT-PCR product

After completion of amplification, the RT-PCR product (5 µl) mixed with 5 µl of gel loading dye, was loaded on 2 per cent agarose gel and electrophoresed in 1X TBE buffer at 100 V for 30-45 min. DNA marker (100 bp DNA lader, Thermo Fisher Scientific) was loaded in one well for size comparison. Before run the gel was stained in ethidium bromide (0.5 mg/µl) and viewed under uv transiluminator and photographed.

3.2.8 Statistical analysis

Statistical analyses were carried out in SYSTAT v12.02 software (SYSTAT Software Inc.) platform with appropriate statistical technique.

3.3 Disease incidence

The incidences of viral diseases were recorded on *A. mellifera* during 2019 to 2020 in the University apiaries located at Chatha, Jammu and at Samba, Ramban, Udhampur. The healthy and diseased samples were collected from experimental colonies and stored in laboratory at 4°C for further investigations.

The severity of the disease with regard to the percent colony infection in the apiary was calculated by using following formulae:

$$\text{Per cent colony infection in the apiary} = \frac{\text{Number of diseased colonies}}{\text{Total number of colonies}} \times 100$$

Chapter-4

Results

CHAPTER-4

RESULTS

The present investigation was carried out at Stress Biology Laboratory of Division of Livestock Production and Management, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-J, R. S. Pura, Jammu, during the year 2019-2020. The results of investigation are presented under suitable headings as under.

4.1 Molecular detection through Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique

The surveyed were conducted in different area of Jammu region viz. Jammu, Samba, Udhampur and Ramban districts. Eight apiaries were surveyed and 600 number of bee colonies were examined for the prevalence of viral diseases. The suspected samples were brought to the laboratory for further studies. The detection of viruses were done through RT-PCR assays of the processed samples of infected *A. mellifera* collected from different districts *viz.,* Jammu, Samba, Ramban and Udhampur. Total RNA was isolated from the infected samples by trizol method. The quality of isolated RNA was checked by resolving RNA on 1.5 per cent agarose gel and has been depicted in Plate 3. The RNA was converted into cDNA by using Hi-cDNA synthesis kit (HI MEDIA). Different pairs of virus specific primers were used for amplifying the viral genome.

The primer pair IAPVVP3RTFW/IAPVVP3RTRV amplified the VP3 region present in the IAPV genome (Fig. 1). The amplicons (amplified product) were observed under UV- transiluminator. The primer pair IAPVVP3RTFW/IAPVVP3RTRV produced the clear and distinct bands of molecular size approximately 110 bp from IAPV infected samples 2 and 4 (Chatha), 5 (Dalwah), 6 (Purmandal), 7 (R. S. Pura), 8 and 9 (Sudhmahadev) (Plate 4). The average cycle threshold (Ct) value of IAPV amplification was 26.65 ± 1.03.

The primer pair KBV3CPRTFW/KBV3CPRTRV amplified the 3C-pro region present in the KBV genome (Fig. 2). The amplicons (amplified product) were observed under UV- transiluminator. The primer pair KBV3CPRTFW/KBV3CPRTRV produced the clear and distinct bands of molecular size approximately 122 bp from KBV infected samples 4 (Chatha), 5 (Vijaypur) and 6 (Dabrah) (Plate 5). The average cycle threshold (Ct) value of KBV amplification was 17.35 ± 2.56.

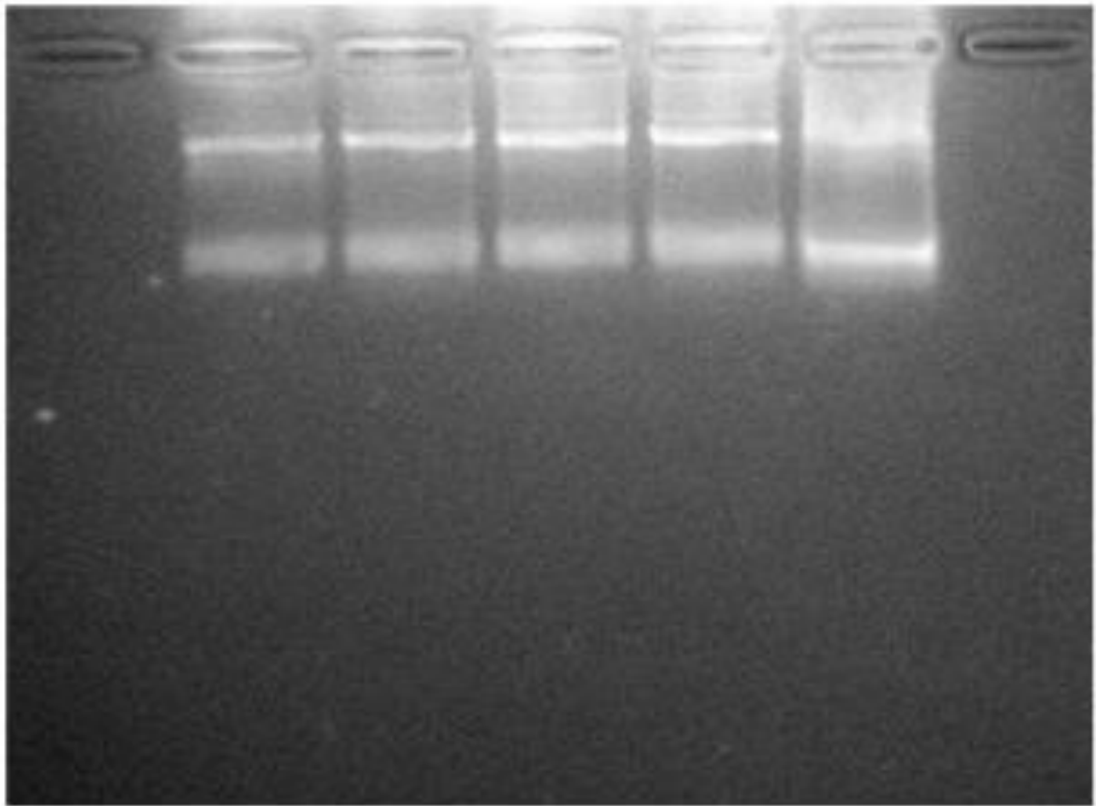
The primer pair SACP1RTFW/SACP1RTRV amplified the polyprotein region present in the SBV genome (Figure 3). The amplicons (amplified product) were observed under UV- transiluminator. The primer pair SACP1RTFW/SACP1RTRV produced the clear and distinct bands of molecular size approximately 119 bp from SBV infected samples 1 (Chatha), 2 (Sudhmahadev) and 3 (Digdol) (Plate 5). The average cycle threshold (Ct) value of SBV amplification was 28.78$\pm$4.01.

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

4.2 Symptomatology

The studies on the symptoms of Israeli acute paralysis, Kashmir bee, sacbrood and Thai sacbrood diseases were conducted in *A. mellifera* during 2019-2020 at University campus, SKUAST - J and survey was also done in four districts Jammu, Samba, Ramban and Udhampur of Jammu region. During the period of investigations, following typical symptoms of the diseases were recorded. The symptoms recorded in Israeli acute paralysis infections include early death of pupae, deformed wings, shortened abdomen and cuticle discoloration in adults bees, which die within 3 days causing the colony to eventually collapse. The honey bees infected with KBV have no reported visible symptoms. The Kashmir bee virus diseases were diagnosed on the basis of molecular characterization of the bee samples (brood and adult workers) collected from the apiaries of different location across Jammu region. The virus was recorded from the mites infested brood adult samples. The sacbrood and Thai sacbrood diseases show typical identifiable symptoms. These diseases killed the brood in prepupal stage and have similar symptoms. During the period of investigations, following typical symptoms of the disease were recorded.

i) Diseased colonies showed mottled appearance and perforated brood capping in combs.
ii) Affected cells were either capped or recently uncapped by cleaner bees. Some punctures, variable in size and in number, were frequently noticed.
iii) Larvae appeared slightly yellow with dark head and had the appearance of a distended sac containing a fluid within a thin cuticle wall.
iv) The prepupae had sac-like appearance which was typical symptom of these diseases.



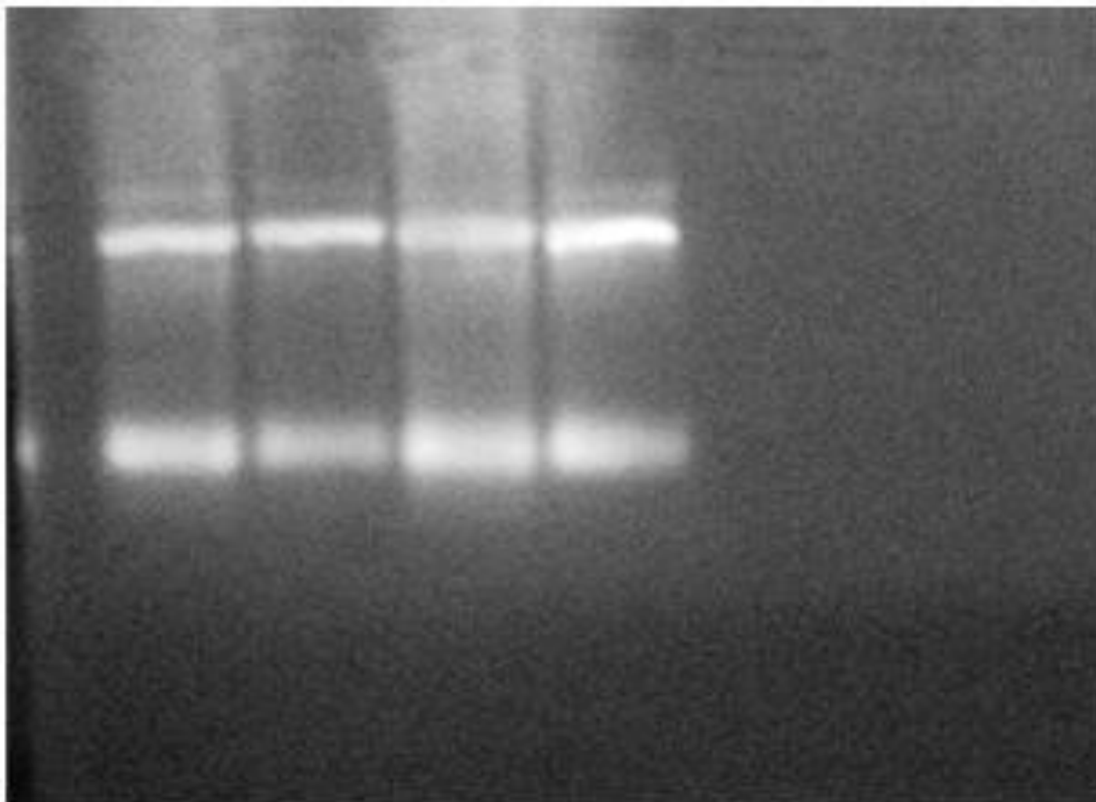


Plate 3: Quality check of RNA on 1.5 per cent agarose gel

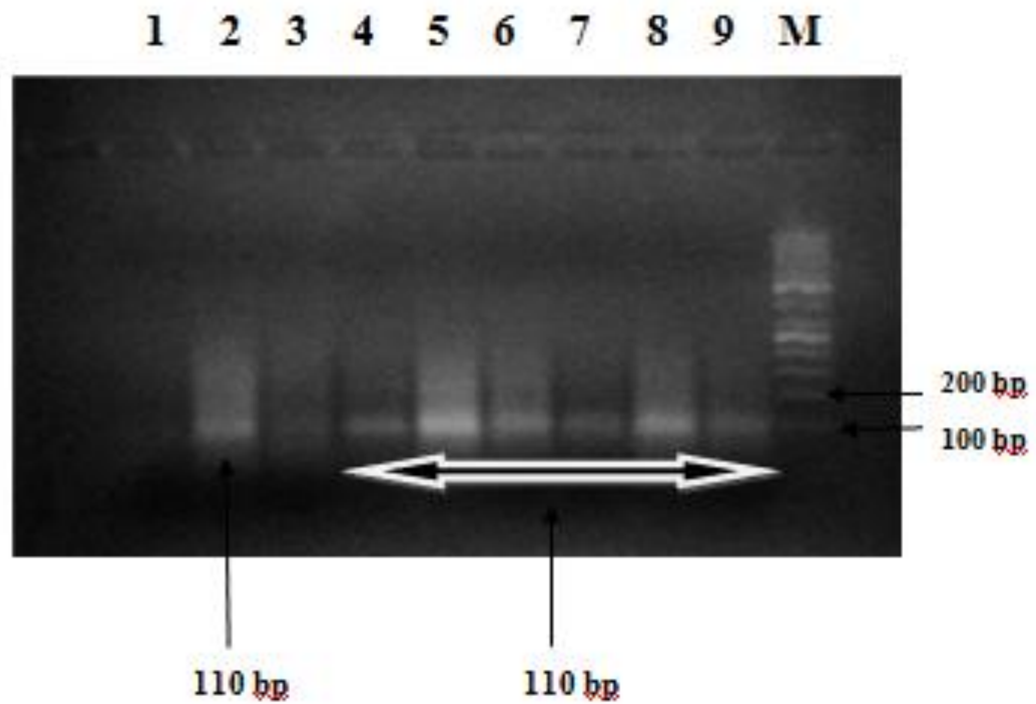


Plate 4: Banding profile of RT-PCR product using IAPV specific primer pair. M: (100 bp ladder), sample 2 and 4 (Chatha), 5 (Dalwah), 6 (Purmandal), 7 (R S. Pura), 8 and 9 (Sudhmahadev): showing IAPV specific band (110 bp), 1 and 3: No amplification

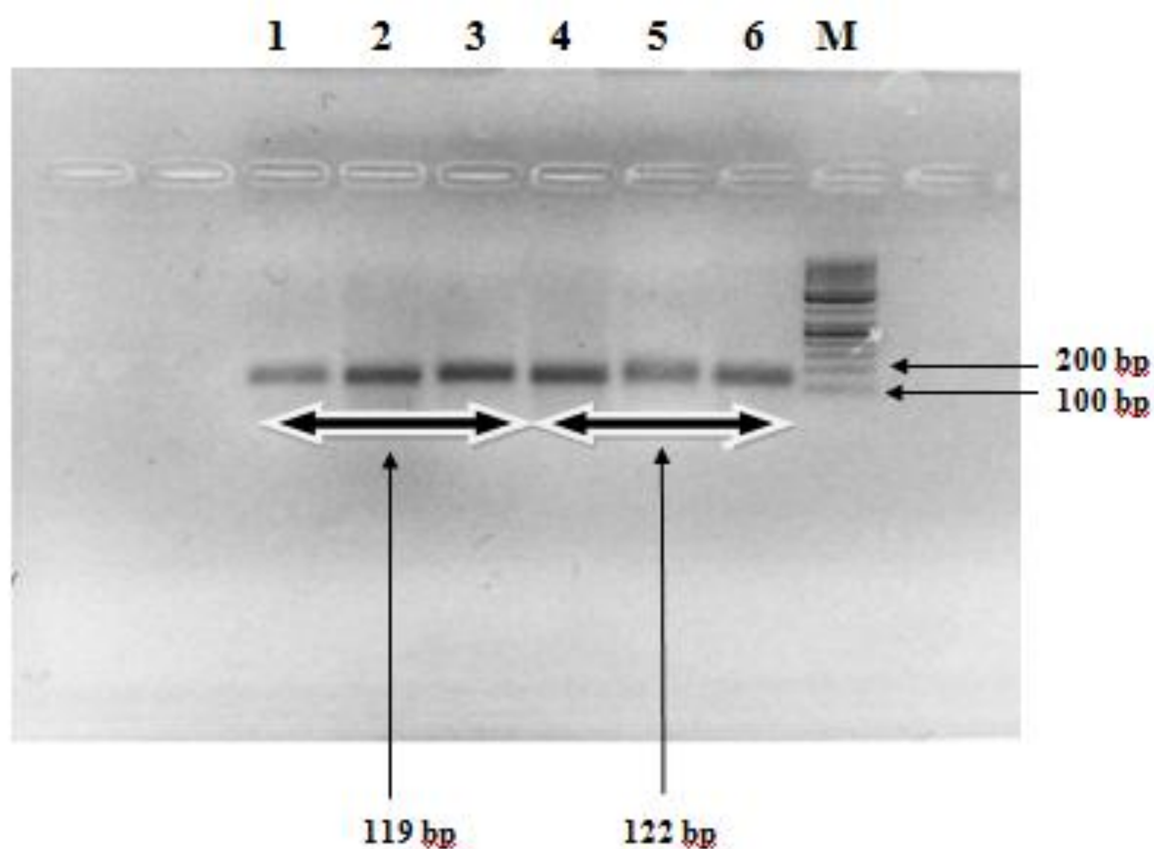
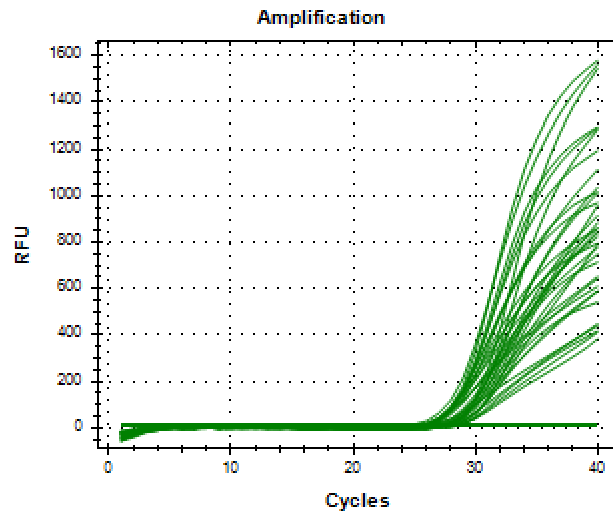
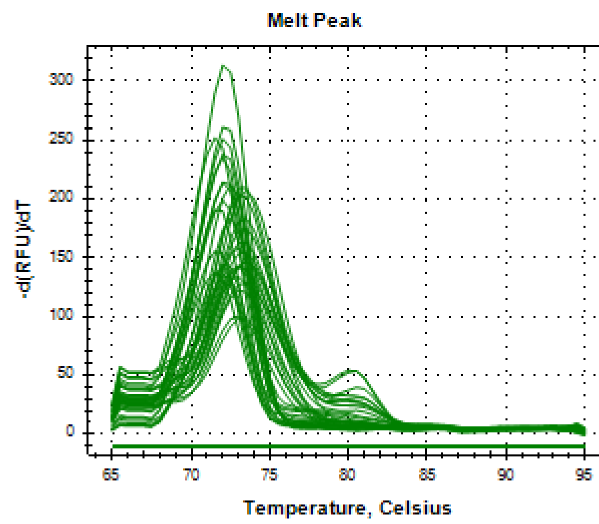


Plate 5: Banding profile of RT-PCR product using KBV and SBV specific primer pairs. M: (100 bp ladder), sample 1 (Chatha), 2 (Sudhmahadev), 3 (Digiol): showing SBV specific band (119 bp) and 4 (Chatha), 5 (Vijaypur) and 6 (Dabrah): showing KBV specific band (122 bp)

A. Amplification Curve



B. Melt Curve



C. Melt Curve

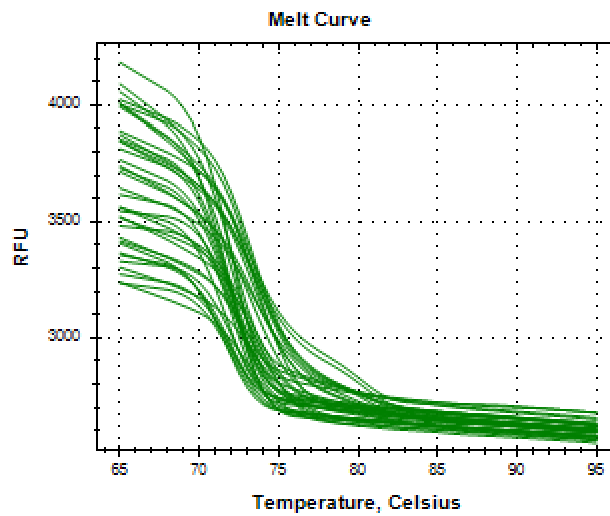
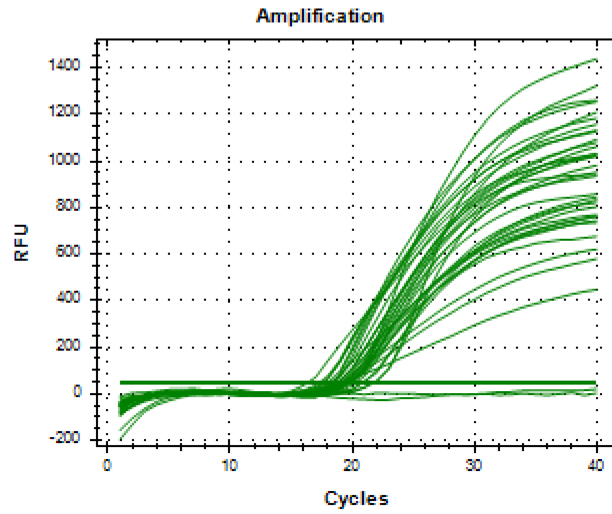
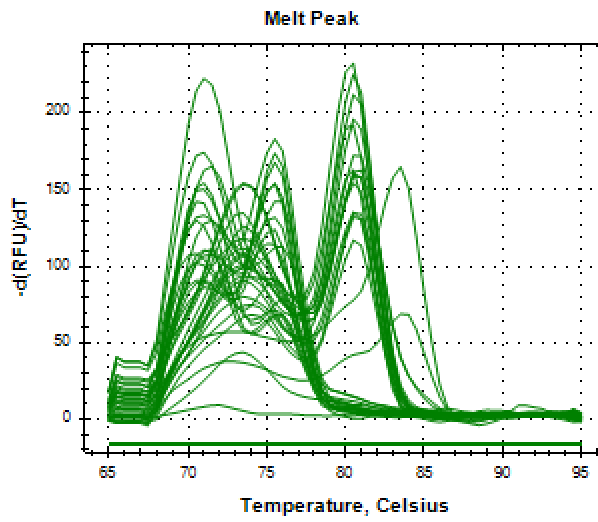


Figure 1: IAPV amplification in RT-PCR with primer set IAPVVP3RTFW/IAPVVP3RTRV

A. Amplification Curve



B. Melt Curve



C. Melt Curve

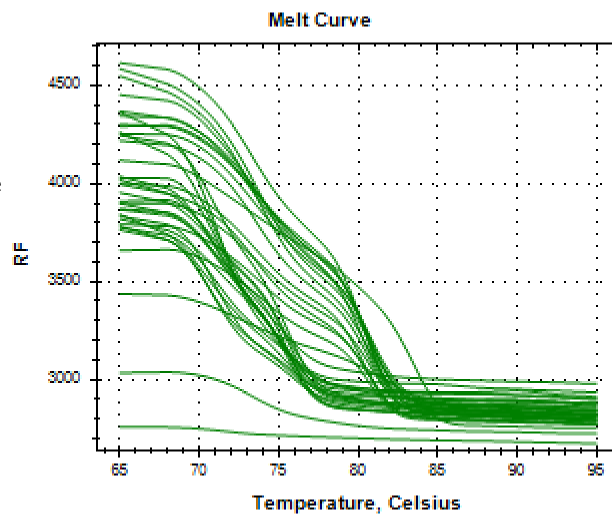
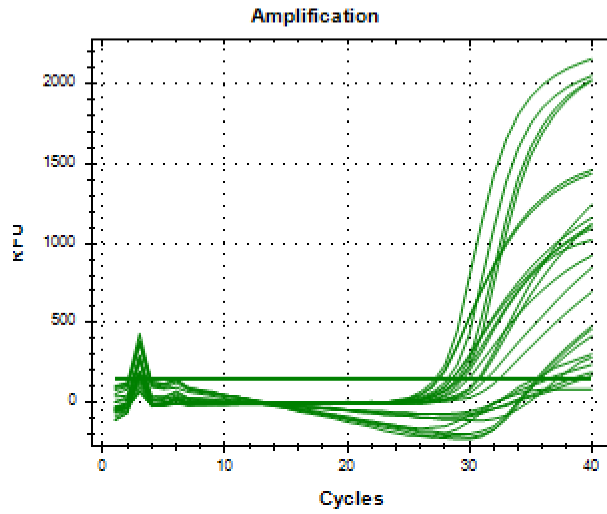
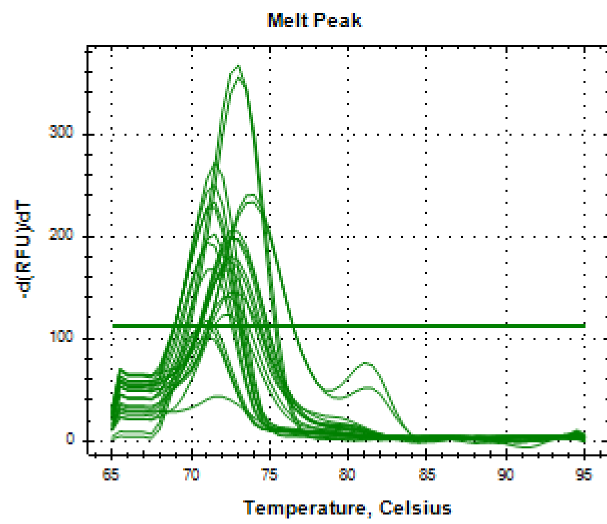


Figure 2: KBV amplification in RT-PCR with primer set KBV3CPRTFW/KBV3CPRTRV

A. Amplification Curve



B. Melt Curve



C. Melt Curve

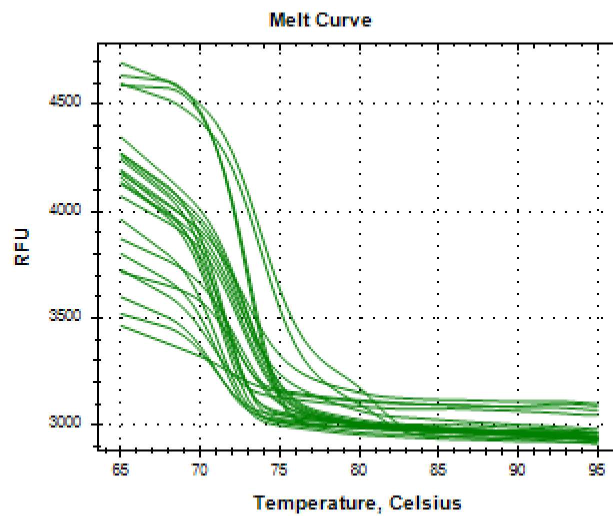


Figure 3: SBV amplification in RT-PCR with primer set SACP1RTFW/SACP1RTRV

v) The brood pattern appeared irregular (scattered), smell-free with punctures on sealed brood.
vi) Slightly yellow to dark brown. Affected un-adherent larvae were easily removed from the cell.

4.3 Disease incidence

The data recorded on the incidence of viral diseases in *A. mellifera* during 2019 to 2020 at Jammu region is given in Table 8 and Fig. 4. The surveyed were conducted in different area of Jammu region viz. Jammu, Samba, Udhampur and Ramban districts. Eight apiaries were surveyed and 600 number of bee colonies were examined for the prevalence of viral diseases. The incidence of Israeli acute paralysis disease caused by Israeli acute paralysis virus was recorded with a minimum of 8.33 per cent in Vijaypur colonies of Samba and maximum 21.42 per cent in Sudhmahadev colonies of Udhampur. Similarly data recorded on Kashmir bee disease caused by Kashmir bee virus and sacbrood disease caused by sacbrood virus in *A. Mellifera* is given in Table 8 and Fig. 4. The incidence of Kashmir bee disease was recorded with a minimum 5.71 per cent in Chatha colonies of Jammu and maximum 28.33 per cent in Vijaypur colonies of Samba. The incidence of Sacbrood disease was recorded with a minimum 8.0 per cent in Digdol colonies of Ramban and maximum 18.75 per cent in Dalwah colonies of Ramban.

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

4.4 Diseases incidence in University apiary

The data recorded on the incidence of viral diseases in *A. mellifera* during July, 2019 to April, 2020 at SKUAST-J apiary is given in Table 9 and Fig. 5. The incidence of Israeli acute paralysis disease was found maximum in October (6.25%) and minimum in November to February (0%). The incidence of Kashmir bee disease was found maximum in September (4.16%) while no incidence of the disease was recorded in surveyed apiaries during the months of December and January. Similarly the incidence of sacbrood disease was found maximum in March (9.43%) and minimum in December to January (0%). The incidence of Thai sacbrood disease was not recorded, as given in Table 9 and Fig. 5.

Table 8: 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				Per cent 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

Table 9: Incidence of IAPV, KBV, SBV and TSBV diseases in *Apis mellifera* in the University apiary

Months	Number of colonies	Number of infected colonies				Per cent 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

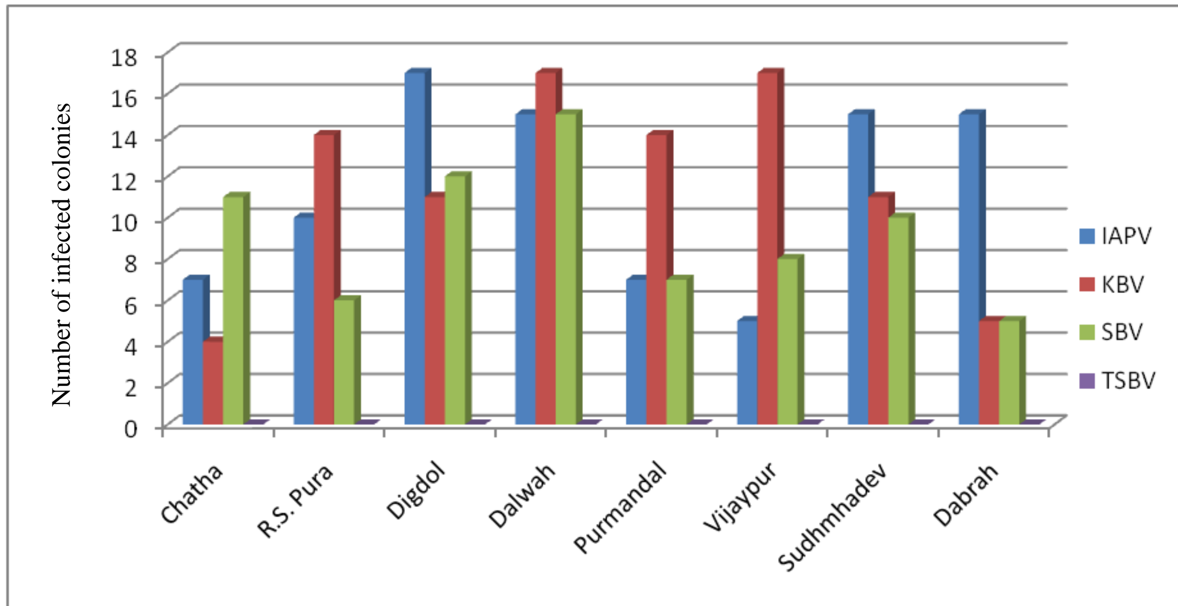


Figure 4: Incidence of IAPV, KBV, SBV and TSBV diseases in the different areas of Jammu region

IAPV- Israeli acute paralysis virus

KBV-Kashmir bee virus

SBV-Sacbrood virus

TSBV-Thai sacbrood virus

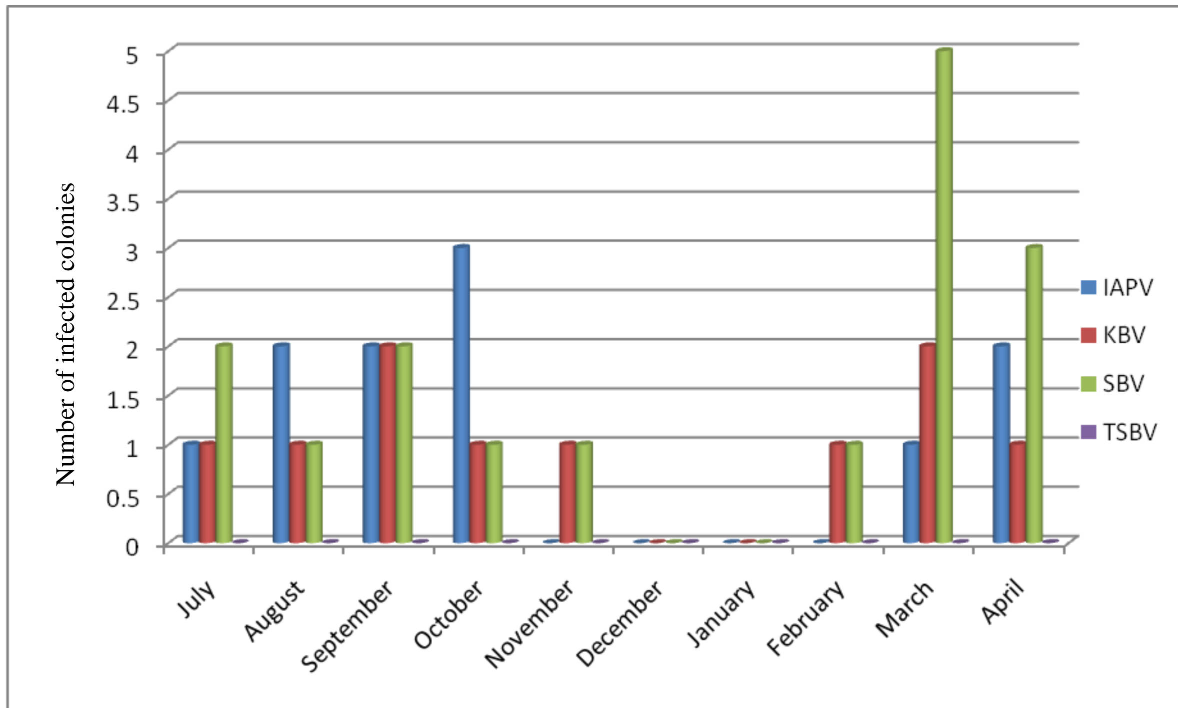


Figure 5: Incidence of IAPV, KBV, SBV and TSBV diseases in *Apis mellifera* during July 2019-April 2020 in the apiary at SKUAST-J, Chatha

IAPV- Israeli acute paralysis virus

KBV-Kashmir bee virus

SBV-Sacbrood virus

TSBV-Thai sacbrood virus

Chapter-5

Discussion

CHAPTER-5

DISCUSSION

Viral bee diseases are major points of consideration in the world economy and India is not an exception. The scientific interest in viral diseases of the honeybee (*Apis mellifera* L.) has been increasing considerably during the past few years. The mortality of honeybees (*Apis mellifera*) is one of the most serious problems that beekeepers have to face periodically worldwide. Although usually not associated with clinical symptoms, viruses in certain cases may cause serious or even lethal disease in individual bees or the collapse of entire colonies. Infestation with the ectoparasitic mite *Varroa destructor* is the major predisposing factor; however, a variety of other weakening circumstances may play a role in clinical manifestation of bee virus infections (e.g., *Nosema apis* infestation, intoxications, environmental pollution, and cold weather) (Suchail *et al.,* 2004). It has also been suggested that episodes of mortality are related to the presence of RNA viruses. The presence of different viruses and their relation with mortality of bees is a cause of concern that is being studied all over the world (Yue and Genersch, 2005).

In practical terms, six viruses are considered to be able to cause severe disease in honeybees and hence they are most important in beekeeping. These are sacbrood virus (SBV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), deformed wing virus (DWV), acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV).

Over 24 stranded positive sense 'picorna-like' RNA viruses have now been characterized as infectious to the European honeybee, *Apis mellifera* L (Table 10). Morphologically, these viruses are similar, exhibiting isometric-shaped protein capsids of approximately 30 nm in diameter. They also share similarities within their genome sequences, particularly within the helicase, protease and polymerase domains of the replicase polyprotein and also with the order of these 3 domains. The newly defined order Picornavirales, often referred to as the Picorna-like super family, encompasses the families Picornaviridae, Dicistroviridae, Comoviridae, Marnaviridae and the Sequiviridae, and the currently unassigned genera, the *Iflavirus*, *Cheravirus*, and *Sadwavirus*. Honeybee viruses of the order Picornavirales include the deformed wing

virus (DWV), acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV), chronic bee paralysis virus (CBPV), sacbrood virus (SBV), black queen cell virus (BQCV), Kashmir bee virus (KBV) and the recently identified Kakugo virus (KV). CBPV remains unassigned, while SBV has been classified as a member of the genus *Iflavirus* and BQCV, KBV and ABPV have been assigned to the family Dicistroviridae. DWV and KV are considered to also be members of the genus *Iflavirus*, however have not yet been formally classified. In addition to the honeybee viruses, a single-stranded RNA virus replicating within *V. destructor* mites, VDV, has now been identified. The VDV genome has now been sequenced and has been shown to be highly similar to DWV and KV, and is therefore tentatively assigned to the *Iflavirus* genus.

For the first time, sacbrood disease of *Apis mellifera* L. was reported from USA in 1917 (White, 1917). Its extent of damage was from a few cells to 90 per cent in brood and 0 to 100 per cent colony infection per apiary in different countries. Thai sacbrood disease of *Apis cerana* Fabricius was recorded from Thailand in 1976 (Bailey *et al.,* 1982). Israeli acute paralysis virus (IAPV) was first described in 2004 in Israel, where severe bee mortality has caused heavy losses to Israeli apiculture (Maori *et al*., 2007). The diseases are still prevalent throughout country and affecting the development and productivity of the beekeeping industry.

In recent years, significant losses of honey bee (*Apis mellifera* L.) colonies have been reported and several studies have connected it to the presence of viruses. Israeli acute paralysis virus (IAPV) has been strongly correlated with a new syndrome of honey bee losses observed in the United States, known as the Colony Collapse Disorder (CCD) (Cox-Foster *et al.,* 2007). ABPV, KBV and IAPV were recently described as members of the 'ABPV complex' of closely related viruses. The original host of ABPV was probably *A. mellifera,* although it was also detected in five bumble bee species (Allen and Ball, 1996). KBV was first detected in *A. cerana* colonies in Kashmir (Bailey and Woods, 1977) and later found to also be infective for *A. mellifera* (de Miranda *et al.,* 2004). IAPV was characterized only recently (Maori *et al.,* 2007) and classified previously as a variant of KBV (de Miranda *et al.,* 2010).

Table 10: Summary of the physical properties of the currently known honey bee viruses

Virus	Physical Properties			
	Shape	**Size**	**Nucleic acid**	**Taxonomy**
Acute bee paralysis virus	icosahedral	30nm	ssRNA	Dicistroviridae
Kashmir bee virus	icosahedral	30nm	ssRNA	Dicistroviridae
Israeli acute paralysis virus	icosahedral	30nm	ssRNA	Dicistroviridae
Black queen cell virus	icosahedral	30nm	ssRNA	Dicistroviridae
Aphid lethal paralysis virus	icosahedral	30nm	ssRNA	Dicistroviridae
Big Sioux River virus	icosahedral	30nm	ssRNA	Dicistroviridae
Deformed wing virus	icosahedral	30nm	ssRNA	Iflaviridae
Varroa destructor virus-1	icosahedral	30nm	ssRNA	Iflaviridae
Egypt bee virus	icosahedral	30nm	ssRNA	Iflaviridae
Sacbrood virus	icosahedral	30nm	ssRNA	Iflaviridae
Thai/Chinese sacbrood virus	icosahedral	30nm	ssRNA	Iflaviridae
Slow bee paralysis virus	icosahedral	30nm	ssRNA	Iflaviridae
Chronic bee paralysis virus	anisometric	30~60 nm	ssRNA	unclassified
Chronic bee paralysis satellite virus	icosahedral	17nm	ssRNA	satellite
Cloudy wing virus	icosahedral	17nm	ssRNA	unclassified
Bee virus-X	icosahedral	35nm	ssRNA	unclassified
Bee virus-Y	icosahedral	35nm	ssRNA	unclassified
Lake Sinai Virus-1	unknown	unknown	ssRNA	unclassified
Lake Sinai Virus-2	unknown	unknown	ssRNA	unclassified
Arkansas bee virus	icosahedral	30nm	ssRNA	unclassified
Berkeley bee picorna-like virus	icosahedral	30nm	ssRNA	unclassified
Varroa destructor Macula-like virus	icosahedral	30nm	ssRNA	Tymoviridae
Apis mellifera filamentous virus	rod	150x450nm	dsRNA	Baculoviridae
Apis iridescent virus	polyhedral	150nm	dsRNA	Iridoviridae

The discussions of present investigation are presented under suitable headings as under:

5.1. Molecular detection through Reverse Transcription polymerase chain reaction (RT-PCR) technique

The use of RT-PCR to detect the RNA viruses in honeybees is a routinely implemented technique and is often coupled with phylogenetic analyses to investigate similarities or differences between virus isolates. Typically, sequences encoding capsid genes and sequences encoding the RNA-dependent RNA polymerase (RdRp) gene have been employed for these studies. In particular, the RdRp is considered a good marker for studies concerning RNA virus classification and evolution, with previous research by Koonin and Dolja identifying 8 conserved domains within the RdRp gene of the positive sense single-stranded RNA viruses. The identified domains are considered to have important functions with respect to RNA polymerase activity, with studies involving amino acid substitutions within particular motifs of these domains having significant impacts on the enzymatic activity.

The confirmation and detection of the causative viruses/organisms, responsible for the diseases in *A. mellifera* were done through RT-PCR.

Based on the observation and results of the study, the virus present in the honeybee samples which caused diseases in *A. mellifera* were detected and confirmed through RT-PCR assay. RT-PCR molecular diagnosis for honeybee viruses were conducted on 200 samples collected from different regions. RNA was isolated from honey bee samples collected at different interval by Trizol method. The RNA was converted into cDNA by using Hi-cDNA synthesis kit (HI MEDIA). Different pairs of virus specific primers were used for amplifying the viral genome.

A total of three honeybee viruses have been identified and all the viruses identified in the study belong to Dicistroviridae and Iflaviridae families. These viruses main feature was positive strand RNA. Most of these viruses have physical features resembling those of picornaviruses, and they are referred to as picorna-like viruses. Of these three viruses, only sacbrood virus causes symptoms that can confidently be attributed to viral infection.

The primer pair IAPVVP3RTFW/IAPVVP3RTRV amplified the VP3 region present IAPV genome. The primer pair IAPVVP3RTFW/IAPVVP3RTRV produced

the clear and distinct bands of molecular size approximately 110 bp from IAPV infected samples. The average cycle threshold (Ct) value of IAPV amplification was 26.65$\pm$1.03.

The primer pair KBV3CPRTFW/KBV3CPRTRV was amplified the 3C-pro region present in the KBV genome. The primer pair KBV3CPRTFW/KBV3CPRTRV produced the clear and distinct bands of molecular size approximately 122 bp from KBV infected samples. The average cycle threshold (Ct) value of KBV amplification was 17.35$\pm$2.56. Present study find support from Francis and Kryger (2012) who used RT-PCR technique for identifying 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.

The primer pair SACP1RTFW/SACP1RTRV amplified the polyprotein region present in the SBV genome. The primer pair SACP1RTFW/SACP1RTRV produced the clear and distinct bands of molecular size approximately 119 bp from SBV infected samples. The average cycle threshold (Ct) value of SBV amplification was 28.78$\pm$4.01. Present study find support from Rana *et al.* (2011) who conducted study on the identification of sacbrood virus through ELISA and RT-PCR technique in diseased prepupae of *A. mellifera* collected from Himachal Pradesh, India. Presence of sacbrood virus was confirmed with 3 primers, *viz.* Sb1f/r, Sb3 f/r and Sb5 f/r. Amplication of the genome produced distinct bands of expected molecular sizes, i.e. approximately 487, 267 and 597 bp. Reddy *et al.* (2017) in a similar study found that sacbrood virus (SBV) samples shared 91-99 per cent identity with each other and shared 89-94 per cent identity with strains from other countries. Studies indicate that host specificity, geographic distance, and viral cross infections between different bee species may explain the genetic diversity among the SBV in *A. cerana* and *A. mellifera* and other SBV strains.

The primer pair TSBVRTFW/TSBVRTRV did not amplify any region present in the TSBV genome. Similar results were also reported by Berenyi *et al.* (2006) in honey bee, they detected Kashmir bee virus (KBV) and sacbrood virus (SBV) with RT-PCR technique. Cagirgan and Yazici (2020) also detected the Israeli acute bee paralysis virus (IAPV), deformed wing virus (DWV), sacbrood virus (SBV) and Kashmir bee virus (KBV) with multiplex RT-PCR (mRT-PCR). The results discussed also confirms the simulation results reported by Ward *et al.* (2007) who developed a real-time

polymerase chain reaction (RT-PCR) assay for specific detection of Kashmir bee virus (KBV). KBV was successfully amplified from different life stages of honey bees. Karapinar *et al*. (2018) determined the presence and prevalence of viral and parasitic infections. The samples were tested by Reverse-Transcriptase PCR (RT-PCR) for acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV) and deformed wing virus (DWV). Selected positives were sequenced, phylogenetically analyzed and investigated in terms of *Varroa*. DWV and BQCV were identified in 69.23 per cent (18/26) and 88.46 per cent (23/26) of the bees respectively whereas ABPV and CBPV were not detected in the sampled apiaries.

5.2 Symptomatology

The present study conducted on the symptoms of Israeli acute paralysis disease in *A. mellifera* found that in *A. mellifera* diseased bee colonies, early death of pupae, deformed wings, shortened abdomen and cuticle discoloration in adults bees, which die within 3 days causing the colony to eventually collapse. IAPV was characterized as a new member of the Dicistroviridae family (Christian *et al*., 2005), closely related to Kashmir bee virus (KBV) and acute bee paralysis virus (ABPV), but genetically and serologically distinct (Maori *et al.* 2007). Recently, the presence of IAPV has been strongly correlated with a new syndrome of honey bee losses observed in the United States, called Colony Collapse Disorder (CCD) (Cox-Foster *et al*., 2007).

Honey bees infected with KBV have no reported visible symptoms. Kashmir bee virus closely related to Israeli acute paralysis virus and acute bee paralysis virus (de Miranda *et al.,* 2010). These findings got support from the findings of Miranda *et al*. (2013) who conducted study on KBV and reported that there was no reported visible symptom and experimental evidence in long-term studies has shown that KBV can be extremely lethal to honey bee larvae and adults.

Sacbrood and Thai sacbrood diseases killed the brood in prepupal stage and have similar symptoms. SBV is present in all the stages of honey bee i.e. 95% of larvae, 80% of pupae and in approximately 85–86% of adult bees (Tentcheva *et al.,* 2004). Affected cells were either capped or recently uncapped by cleaner bees. Some punctures, variable in size and in number, were frequently noticed. Larvae appeared slightly yellow with dark head and had the appearance of a distended sac containing a fluid within a thin cuticle wall. The accumulated liquid in the sac consisted of a more or less granular appearing mass suspended in a watery fluid. The brood pattern appeared

irregular (scattered), smell- free with punctures on sealed brood. Affected larvae appeared slightly plumpy compared to healthy ones when examined by taking out of the infected cells. Infected larvae were seen stretched on their back in the cells with the head directed outwards and turned upwards like the prow of a boat. The dead larvae looked like a sac filled with milky white fluid when lifted up and it ruptured easily releasing the milky fluid. Cadavers changed their colour from white to pale yellow and sank down on the floor of the cell and dried up in 10-15 days as brownish black boat like cells. Sequences of visible symptoms in infected colonies were as follows: Presence of unsealed cells in brood area with the head of the larvae directed outwards like the prow of a boat. Dead larvae lying stretched out on their back appearing like sacs filled with milky white fluid when lifted up. These findings are in line with the findings of Devanesan and Jacob (2001), they reported that one day old larvae of Asian honeybee *Apis cerana indica* Fab. were highly susceptible to Thai sac brood virus recording 100 per cent mortality closely followed by 2 and 3 day old larvae showing 84 to 92 and 82 to 96 per cent mortality, respectively. Four day old larvae were comparatively less susceptible recording 70-74 per cent mortality only. Berenyi *et al.* (2006) also reported the prevalence of SBV.

5.3 Disease incidence

The viral honey bee diseases are an increasing beekeeping problem throughout the world due to great economic losses and a reduction in biodiversity in natural ecosystems (Gajger *et al.,* 2014). In the present study, it was observed that the incidence of Israeli acute paralysis disease was recorded with a minimum of 8.33 per cent in Vijaypur and maximum 21.42 per cent in Sudhmahadev. The incidence of Kashmir bee disease was recorded with a minimum 5.71 per cent in Chatha and maximum 28.33 per cent in Vijaypur. The incidence of sacbrood disease was recorded with a minimum 8.0 per cent in Digdol and maximum 18.75 per cent in Dalwah. The incidence of Thai sacbrood disease was not recorded during the year 2019-2020 in any of the surveyed region. The incidence of sacbrood disease in the University apiary was found maximum in March (9.43%) and minimum in December to January (0%).The incidence of Thai sacbrood disease was not recorded during July, 2019 to April, 2020. The sacbrood virus is a single-stranded, positive sense RNA virus belonging to the genus Iflavirus in the family Iflaviridae. The virus primarily affects the brood stage of honey bees causing failure of pupation and larval death but, also affects adult bees

without obvious signs of the disease. Sacbrood is characterized by the diseased infected larva, encased in a tough, fluid filled sac formed from the larval cuticle (Gong *et al.,* 2016). The SBV has been reported to prevalent in *A. mellifera* bees from Denmark, France, and Austria (Berenyi *et al*., 2006; Nielsen *et al*., 2008; Tentcheva *et al*., 2004). However, this virus was more prevalent in *A. cerana* than *A. mellifera* in China. SBV caused serious Chinese sacbrood disease in *A. cerana* (Yan *et al*., 2009). The sacbrood virus killed honey bee brood at the prepupal stage (10 days of age) on the second day after the sealing of brood. Sacbrood virus was detected in colonies during spring and summer (March to May) affecting 0.39 per cent to 5.20 per cent of the brood (Rana and Rana, 2008). Freiberg *et al*. (2012) in a similar study also reported that sacbrood of *A. mellifera* characterized by the brood that failed to pupate and subsequently died, the decline was reported as an important threat to honey bee health. Rana and Rana (2015) reported that the virus killed honey bee brood at the prepupal stage (10 days of age) on the second day after the sealing of brood. SBV was detected at two locations in Himachal Pradesh, Solan district, Nauni, it was detected in colonies during spring and summer (March to May) when it affected 0.39 per cent to 5.20 per cent of the brood. At the second location, Kangra district, Jachh (Himachal Pradesh), the disease was also detected during spring and summer (March to June) when it affected 0.23 per cent to 2.1 per cent of brood. The incidences of the disease were found to be significantly correlated with colony strength and brood rearing.

In the present study, it was observed that the incidence of viral disease in SKUAST-J apiary from July, 2019 to April, 2020. The incidence of Kashmir bee disease was found 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 Jammu region of Jammu and Kashmir. Nielsen *et al.* (2008) also observed the presence of 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) with RT-PCR. They detected six types of viruses with significantly varied frequencies: emphasizing that SBV was detected in 78 apiaries, DWV in 55 apiaries, ABPV in 11 apiaries, CBPV in 4 apiaries, BQCV in 1 and KBV in 1 apiary. The results discussed also confirms the simulation results reported by Todd *et al.* (2007) who studied on the virus status of honey bee colonies infested with *Varroa destructor.* They

observed viruses *viz.,* CBPV, BQCV, SBV, DWV and KBV during the study, which DWV and KBV were the most common viruses.

The present study reported the incidence to Israeli acute paralysis virus (IAPV) from University apiary of SKUAST-J. The incidence of Israeli acute paralysis disease was found maximum in October (6.25%) and no disease incidence was recorded from November to February (0%). Israeli acute paralysis virus (IAPV) is associated with colony collapse disorder of honey bees. Nonetheless, its role in the pathogenesis of the disorder and its geographic distribution are unclear (Palacios *et al.*, 2008). Ai *et al.* (2012) in a similar studies reported prevalence of seven honey bee viruses not only from *A. mellifera* but also from *A. cerana* apiaries in China. Six viruses except KBV were detected from *A. mellifera*. DWV was found to be the most prevalent, present from 9 per cent of the apiaries. BQCV, SBV, IAPV, ABPV and CBPV were present in 44 per cent, 21 per cent, 18 per cent, 6 per cent and 9 per cent of the apiaries, respectively. The virus prevalence from the apiaries of *A. cerana* was quite different. SBV was the dominant and present in 86 per cent of the apiaries, followed by DWV present in 64 per cent of the apiaries, and IAPV, CBPV or BQCV present in 7 per cent of the apiaries, respectively. Neither KBV nor ABPV were detected from *A. cerana* populations. KBV was not present in any of the samples of two honeybee species in China. This result agreed with another survey in Austria in 2004 (Berenyi *et al*., 2006). This virus was widespread in the United States and Australia (Allen and ball, 1996), but so far it was detected only in few colonies in European countries (Nielsen *et al*., 2008). IAPV was found in 18 per cent of samples in *A. mellifera* from five provinces (Fujian, Zhejiang, Helongjiang, Guangdong and Sichuan), it was also detected in one apiary of *A. cerana* in Guangdong. So far, the only known host of IAPV was *A. mellifera* (de Miranda *et al*., 2010). IAPV was first detected in *A. mellifera* in Shaoguan, Guangdong province in China in 2008 (Yan *et al.,* 2009). But IAPV was first detected in *A. cerana*. They further reported that that the newly emerging virus of IAPV has expanded its host from *A. mellifera* to *A. cerana.* Rustemoglu *et al.* (2019) reported the occurrence and prevalence of black queen cell virus (BQCV), deformed wing virus (DWV), sacbrood virus (SBV), chronic bee paralysis virus (CBPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV). The presence of BQCV, DWV, SBV and CBPV infections were ascertained for the first time by RT-PCR. BQCV was the most frequently detected virus, found in 32 per cent of bee samples, less than 24 per cent of

asymptomatic bee samples were positive for DWV, SBV and CBPV with the infection ratio of 23, 12 and 9 per cent, respectively. During this survey, KBV and IAPV were not detected in any of tested bee samples. Some double and triple infections were encountered: 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%).

Chapter-6

Summary and Conclusion

CHAPTER-6

SUMMARY AND CONCLUSIONS

The present study was undertaken to study the nucleic acid based identification and diversity study of major honeybee (*Apis mellifera* L.) viruses in Jammu region with an aim to develop nucleic acid based detection assay for detection of honeybee viruses.

The virus present in the larval and adult samples which caused diseases in *A. mellifera* were detected and confirmed through RT-PCR assay. The RT- PCR assay was able to detect the Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV) and sacbrood virus (SBV) with the help of specific primers.

The primer pair IAPVVP3RTFW/IAPVVP3RTRV amplified the VP3 region present in the IAPV genome and produced the clear and distinct bands of molecular size approximately 110 bp from IAPV infected samples. The average cycle threshold (Ct) value of IAPV amplification was 26.65±1.0. The primer pair KBV3CPRTFW/KBV3CPRTRV amplified the 3C-pro region present in the KBV genome and produced the clear and distinct bands of molecular size approximately 122 bp from KBV infected samples. The average cycle threshold (Ct) value of KBV amplification was 17.35±2.5. The primer pair SACP1RTFW/SACP1RTRV amplified the polyprotein region present in the SBV genome and produced the clear and distinct bands of molecular size approximately 119 bp from SBV infected samples. The average cycle threshold (Ct) value of SBV amplification was 28.78±4.01. The primer pair TSBVRTFW/TSBVRTRV did not confirm the TSBV in any of the sample.

The symptoms of Israeli acute paralysis diseases were recorded include: early death of pupae, deformed wings, shortened abdomen and cuticle discoloration in adults bees, which die within 3 days causing the colony to eventually collapse. There was no reported visible symptom of KBV infection. The symptoms of sacbrood and Thai sacbrood diseases were recorded include: affected cells were either capped or recently uncapped by cleaner bees and the prepupae had sac-like appearance which was typical symptom of these diseases.

The present study indicated that the incidence of Israeli acute paralysis disease was recorded with a minimum in Vijaypur (8.33%) and maximum in Sudhmahadev (21.42%). Similarly, the incidence of Kashmir bee disease was

recorded with a minimum in Chatha (5.71%) and maximum in Vijaypur (28.33%). The incidence of sacbrood disease was recorded with a minimum in Digdol (8.0%) and maximum in Dalwah (18.75%). The incidence of Thai sacbrood disease was not recorded during the year 2019-2020 in any of studied region.

In the present study, it was recorded that the incidence of viral diseases in SKUAST-J apiary from July, 2019 to April, 2020. The incidence of Israeli acute paralysis, Kashmir bee and sacbrood diseases were found maximum in October (6.25%) and minimum in November to February (0%), maximum in September (4.16%) and minimum in December to January (0%) and maximum in March (9.43%) and minimum in December to January (0%), respectively. The incidence of Thai sacbrood disease was not recorded during July, 2019 to April, 2020.

Conclusions

Three types of viruses were confirmed by RT-PCR Diagnosis (IAPV, KBV and SBV) in the Jammu region of Jammu and Kashmir.

The Israeli acute paralysis, Kashmir bee and sacbrood diseases incidence ranged from 8.33 to 21.42 per cent, 5.71 to 28.33 per cent and 8.0 to 18.75 per cent, respectively in the Jammu region.

The Israeli acute paralysis, Kashmir bee and sacbrood diseases incidence ranged from 0 to 6.25 per cent, 0 to 4.16 per cent and 0 to 9.43 per cent, respectively during July, 2019 to April, 2020 at SKUAST-J apiary.

The RT-PCR technique has provided accurate, rapid and sensitive technique for diagnosis of honey bee diseases of viral origin.

Surveillance and timely collection of samples from *A. mellifera* colonies could prove the efficient diagnosis and management of diseases.

It can be concluded that Jammu and Kashmir is contiguous in borders with Pakistan, China, Russia and Afghanistan having likelihood of obtaining honeybee diseases more often than not from the adjoining countries and can be a threat for the rest of the world and especially for Europe, as a potential centre of spreading diseases and parasites. Hence determination of diseases in this part of the world is of paramount importance to provide safety to bees in India and elsewhere.

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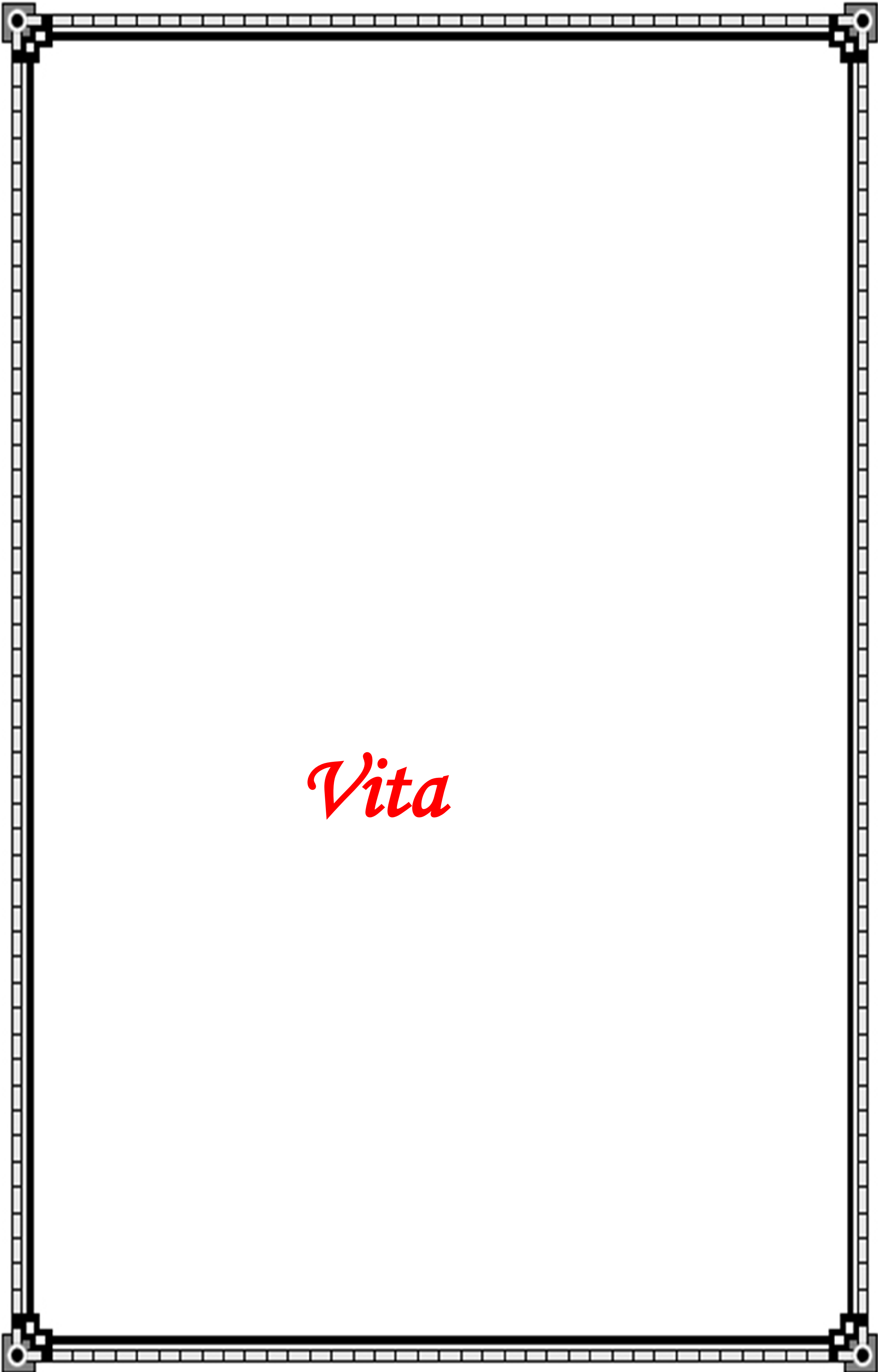

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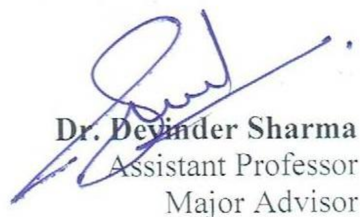
CERTIFICATE-IV

Certified that all the necessary corrections as suggested by the external examiner and the Advisory Committee have been duly incorporated in the thesis entitled **"Nucleic acid based identification and diversity study of major honeybee (*Apis mellifera* L.) viruses in Jammu region"** submitted by **Mr. Ram Narayan Sharma**, Registration No. **J-18-M-550**.



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