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Molecular detection and seasonal variation of deformed wing virus affecting honey bee, *Apis mellifera* L. colonies

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Abstract
A survey was conducted on a geographic scale of Kashmir valley for the detection of Deformed Wing Virus (DWV) using RT-PCR and specific primers. Samples of adult bees and pupae were collected from 20 apiaries in 5 different locations in the spring, summer and autumn seasons were screened for the presence of DWV during 2012-13. In the present investigation, Deformed Wing Virus (DWV) was found in 80% 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. The recorded DWV frequency in the spring and summer was 37 and 65% respectively, but in autumn, it was 32%. In pupae, it was 16, 26 and 7% respectively. As a whole, higher virus frequency was detected in brood populations (92%) than adult populations (90%). The present findings support the role of *Varroa* mite in transmitting the virus. This was the first report on detection of DWV in honeybee colonies in Kashmir valley. Taken together, the data indicated that DWV occur persistently in bee colonies since 2005.

Keywords: Honeybee**,** *Apis mellifera* L., Deformed Wing Virus, Varroa mite, RT-PCR,

1. Introduction
The beekeeping industry plays a key role in temperate fruit production (especially apple production) in Kashmir Valley. Honey bee, *Apis mellifera* L. is an economically important beneficial insect that assists in the pollination of a wide variety of crops with an annual added market value exceeding 15 billion dollars [1]. A large proportion of beekeepers complained of considerable bee mortality in their hives due the prevalence of ectoparasitic mite, *Varroa destructor* since 2005. Mass mortality of honeybees (*Apis mellifera*) due to *V. destructor* is one of the major problems that the beekeepers and the dependent industries face worldwide. To date, approximately 18 honeybee viruses have been identified, which are distributed worldwide [2, 3, 4]. They usually cause unapparent infections and may not be perceived by beekeepers for many years. One of the principal causes of colony collapse is the rapid dissemination of the ectoparasitic mite, *V. destructor* in *A. mellifera* L. colonies [5]. Deformed Wing Virus (DWV), a positive stranded RNA virus, is very common virus infecting honeybee adults and larvae [6, 7]. This virus is reported from almost all *V. destructor* infested hives and presence of wingless adult bees in a colony is accepted as an indicator of this virus [8, 9]. Infected mites play a significant role in transmission of the virus among honeybee colonies [9, 10]. Furthermore, it is the level of DWV present in the bees that determines whether they are deformed at emergence, rather than just the presence or absence of virus [7]. Since wing deformities and DWV infection are associated with *Varroa* infestation [11, 12] and symptoms become apparent by the presence of the *Varroa* mite, because the parasitic mite, *Varroa destructor* feeds and moves regularly between brood and adult bees. These mites have the potential to act as either biological or mechanical vectors of bee viruses. This mite is now widespread in Europe, North Africa, Asia, and Africa, and colony death occurs in 2 years if the mite infestation is not controlled by acaricide treatments. The collapse of bee colonies severely infested by *V. destructor* has long been attributed to viruses and has been described as bee parasitic mite syndrome [13]. We report here the first survey for the detection and seasonal variations of DWV in various apiaries in Kashmir Valley based on sampling of adult bees having different geographic origins. In this study, we use molecular techniques to evaluate the ability of *V. destructor* to transmit DWV and present evidence that these mites are capable of transmitting virus through their feeding habits survived on bee brood in spring and summer

and adult bees in autumn.

2. Materials and Methods

2.1 Collection of samples and extraction of total RNA

Samples of adult worker bees and pupae were collected from 100 infested colonies originating from 20 widely separated apiaries of 5 locations of Kashmir. Five adult bees and pupae were collected individually from each colony following the method described by [3]. The samples were collected in three seasons; spring (March to May), summer (June to August) and autumn (September to November) during 2012-13. Throughout the collection of adult bees and pupae, a visual observation of mite infestation in each colony was made and noted. Total RNA was extracted from adult bees and pupae individually using Trizol reagent (Biobasic) according to the recommendation of manufacturer's protocol. Samples were homogenized by using pestle and mortar with 1 ml Trizol reagent (Biobasic) and transferred to 1.5 ml Eppendorf tubes. The protein fraction was separated out in the organic (lower) phase using chloroform and the RNA was precipitated from the aqueous (upper) phase with 1 ml of iso-propanol by centrifugation (at 12000 rpm for 10 minutes). The resultant nucleic acid pellets were washed twice with 70% ethanol and resuspended in nuclease-free water. The samples were stored at -80^{0}C for further analysis.

2.2 RT-PCR amplification

Each RNA sample was analyzed for the presence of DWV using the Access RT-PCR system (Promega, USA) according to the manufacturer's protocol. DWV specific primer pairs (5'-CTTACTCTGCCGTCGCCCA-3', 5'-CGTTAGGAACTCATTAT CGCG-3') based on [14] were used to amplify a 194bp RNA fragment. Amplification occurred in a 25 µl reaction mixture containing 1X AMV/Tfl reaction buffer, 0.2 mM each dNTP, 1 µM of each primer, 2 mM MgSO4 , 0.1 unit AMV reverse transcriptase, and 0.1 unit Tfl DNA polymerase and 250 to 500 ng of total RNA. The RT-PCR was performed under the following conditions: one cycle at 48 °C for 45 min; 95 °C for 2 min; 40 cycles at 95 °C for 30 s, 55 °C for 1min, and 68 °C for 2 min; 68 °C for 10 min. PCR products of 2-4 µl were analyzed on a 2% agarose gel containing ethidium bromide, visualized on a UV transilluminator (Syngene, UK) and photographed using a digital camera (Sony, Japan). Fragment sizes were determined with reference to 100-bp ladder (Promega). After the analyses, colonies in which adult bees and pupae had virus were designated by DWV positive. Colonies without detectable virus were designated by DWV negative.

3. Results and Discussion

RT-PCR was carried out with each of the primer pair specific to generate PCR fragments of 194 bp (Fig. 1). Out of 100 colonies examined, 71% were found to be infected with DWV. This indicated that DWV is the most common virus in collapsing bee colonies in Kashmir. The prevalence of the DWV in bee colonies is shown in (Table 1).

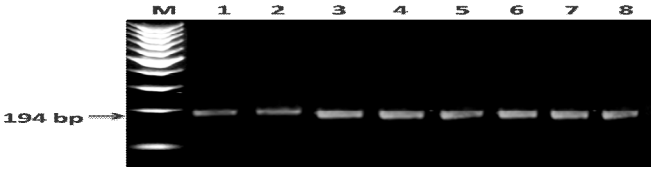

Fig 1: Detection of DWV in representative honey bee colonies.

LaneM:100 bp DNA ladder; Lanes 1, 2, 3 and 4:RNA isolated from adult bees of the colony; Lanes 5, 6, 7 and 8: RNA isolated from pupae of the colony.

Table 1: Prevalence of DWV in honey bee, *Apis mellifera* samples from Kashmir valley

Apiary Number	Apiary	Location	Adult			Pupae		
			SP	SU	AU	SP	SU	AU
AN01	Badroo	Anantnag	+	+	+	+	+	+
AN02	Ranipora		+	+	+	+	+	+
AN03	Verinag		-	-	-	-	-	-
AN04	Sarnal		+	+	+	+	+	+
PU05	Tral	Pulwama	+	+	+	+	+	+
PU06	Midroo		+	+	+	+	+	-
PU07	Lurgam		+	+	-	-	-	-
PU08	Tahab		-	+	+	+	+	+
SH09	Zainpora	Shopain	+	+	+	+	+	+
SH10	Sedaw		+	+	-	+	+	+
SH11	Kachdoor		-	-	-	-	+	-
SH12	Shopain		+	+	+	-	+	+
KU13	D.H. Pora	Kulgam	+	+	+	+	-	-
KU14	Qazigund		+	+	+	+	+	+
KU15	Yaripora		-	+	-	-	-	-
KU16	Dewsar		+	-	-	+	+	-
BA17	Ajas	Bandipora	+	+	+	+	+	-
BA18	Arin		-	+	+	-	+	+
BA19	Chuntimula		+	+	-	+	+	-
BA20	Malangam		+	+	+	+	+	-
Percentage (%) of Positive apiaries			15 **(75)**	17 **(85)**	13 **(65)**	14 **(70)**	16 **(80)**	10 **(50)**

SP = Spring, SU= Summer, AU= Autumn, (+) = Positive; (-) = Negative;

As a whole, higher virus frequencies were found in adult populations (75%) than in brood populations (66.66%) (Fig. 2). No significant differences were found between the locations on the basis of viral infection. Although viral infections were detected during the year, some seasonal variation in virus frequencies was observed in both adults and pupae. DWV infections increased from spring to summer and decreased in autumn for both adults and pupae. The less brood conditions and low temperature in autumn decrease the virus infection rate in bee colonies. The percentage of positive apiaries in the spring, summer and autumn was 75, 85 and 65% respectively. However, in pupae, the percentage in the spring, summer and autumn was 70, 80 and 50% respectively (Fig. 3).

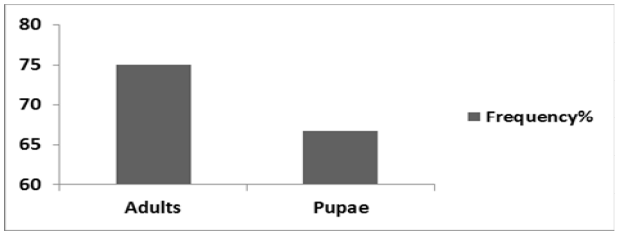

Fig 2: Frequency of DWV in bee colonies from Kashmir valley

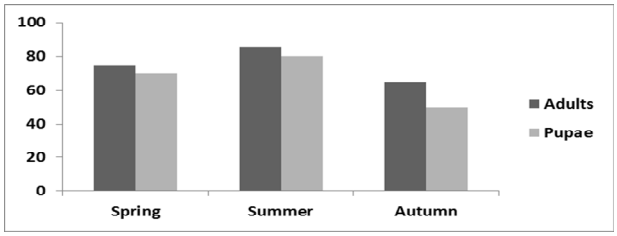

Fig 3: Percentage of DWV infection in different seasons

DWV was most frequently detected virus through PCR in honeybee colonies in adults and pupae. For adults and pupae, the frequency of DWV infected colonies increased from spring to summer but decrease in autumn. The seasonal variations in DWV incidence were much more pronounced for adult bees than for pupae. Another study on bee virus [15] reported that 98% of the bee samples were infected with DWV and the data confirm the putative role of *Varroa* in the transmission of this virus. Interestingly, a large number of DWV positive colonies were detected both for adult bees and for pupae. DWV is thought to be responsible for wing deformities, when infection occurs during the white eyed pupation stage of bee development [6]. DWV has been detected by RT-PCR in the United Arab Emirates [16] although; no other viruses were surveyed in these studies. Further research is needed to determine which other factors are also differentially associated with colony mortality, such as infestation with parasitic mites (*Varroa destructor*, *Acarapis woodi*), *Nosema apis* and *N. cerana* [17], bacterial diseases and any possible effects of chemical treatment on colonies or foraging resources. The present study contributes in increasing the awareness among beekeepers, bee researchers and bee dependent industries in Kashmir region that the diseases and parasites that threaten the bee industry world-wide are similarly present in Kashmir region associated with bee colony mortality. However, the mechanisms that lead to these symptoms are not clearly understood, and usually only a few bees in a colony severely infested by *V. destructor* display such deformities. DWV has been cited as potentially responsible for bee colony collapse [18], but other studies have shown that this virus might be considered poorly pathogenic [7]. Bees could also develop a kind of molecular defence mechanism to reduce virus multiplication. These results provide clear evidence that DWV infection occurs persistently in bee colonies, despite the lack of clinical signs. The frequency of mite infestation suggested that *V. destructor* probably contributes efficiently to the outbreak of bee virus diseases, acting both as a vector and as an activator of virus replication. The latter phenomenon is a very challenging field for understanding the relationships among virus, bees and *V. destructor*.

Several reports on the presence of honey bee viruses in *A. mellifera* L. populations in different countries have been published [19,10,20,21,9,22], some of them before the spread of *V. destructor* in Europe. Most of these reports were based on symptomatic or dead bees collected at the hive entrance, sometimes after colony collapse; i.e., most of the time the data were obtained from a few samples that were not representative of the natural occurrence of virus infections in bee colonies. Here, we describe the first survey of the prevalence of DWV in seemingly affected bee colonies randomly chosen from various apiaries of Kashmir valley. Presently, the beekeepers of the region are facing a big challenge since 2005 to control *V. destructor*. This mite can cause direct as well as indirect loss to the beekeepers either through reduction in the number of colonies or through less honey production.

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