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in SBV frequencies observed both in adult bees and in pupae might reflect a difference in bee susceptibility to the virus or to changes in the environment, such as the quality of the pollen consumed by larvae. Bees could also develop a kind of molecular defense mechanism to reduce virus multiplication. However, the putative role of varroa cannot be excluded in SBV propagation as the virus was detected in few samples of varroa collected from bee colonies in the studied districts (Tentchev et. al. 2004b) .

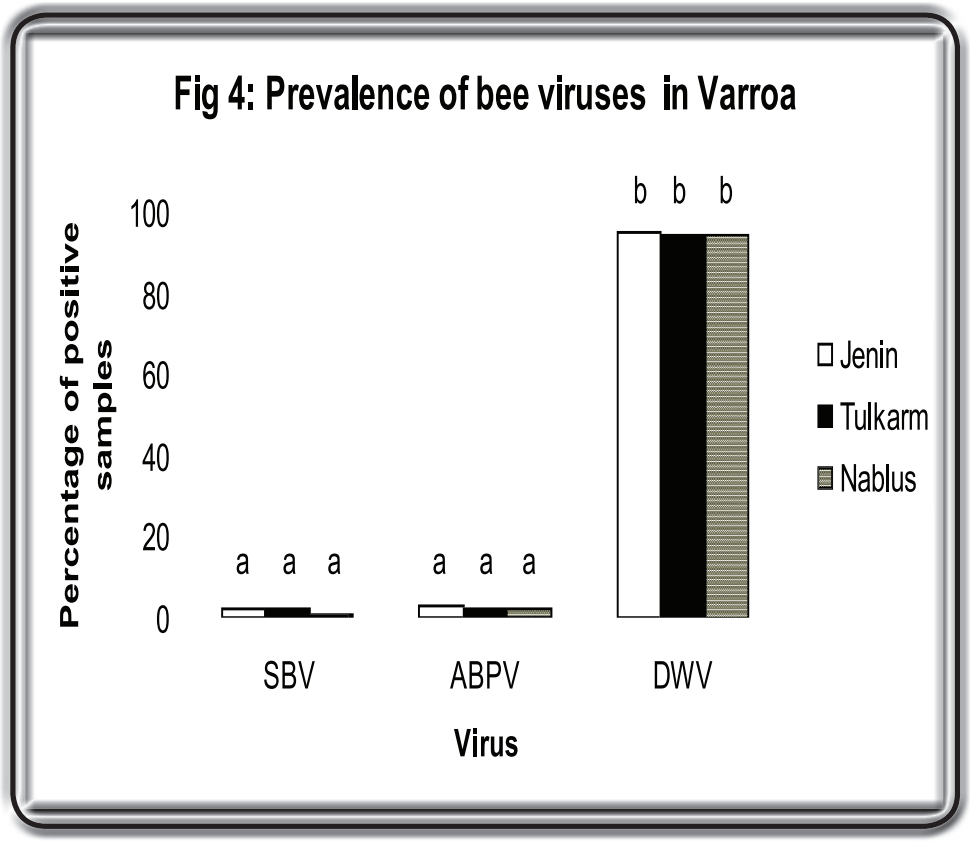
In Jordan, Haddad et. al. (2008) reported that ABPV, SBV and DWV were the most important viruses that cause mortality of honey bees and create periodical serious problems for the beekeepers in Ajlon area. In addition, the author reported that out of thirteen colonies examined, 92% were infected with DWV, 8% with SBV and 16% with ABPV.

Our data show that virus infections in apiaries are quite common in Palestine. Besides, multiple infections were the principle disease in all the apiaries, and no significant geographical localization could be established for a given virus. These findings give an obvious indication that bee virus infections happen steadily in bee colonies, despite the absence of clinical signs. One of the consequences of this is that the spread of virus disease leading to colony collapse probably results from external factors that provide suitable conditions. One of these is the spread of the virus between honeybees of the same colony and between different colonies. A second possible condition is the dissemination and replication of a virus from primary infection sites (e. g., gut epithelial cells) to critical targets (e. g. , the nervous system) in bees. In addition, the absence of virus infection from samples collected from certain locations may be related to the geographical isolation of this apiary, It is worth noting that this finding correlates very well with the lack of varroa mites in this area. The high occurrence of viruses in mites proposes that varroa probably contributes actively to the outbreak of bee virus diseases, acting both as a vector and as an activator of virus replication. The latter finding is a very questioning field for better understanding of the relationships among viruses, honey bee colonies, and varroa.

Discussion:

Of the three viruses identified by RT- PCR in this research, DWV was by far the most frequently detected in colonies, both in bee samples (adults and brood) and in varroa mites. This indicates that DWV is the most common virus responsible for injuring bee colonies in Palestine. For adults and pupae, the frequency of DWV- infected colonies increased from spring to autumn. The seasonal variations in DWV incidence were much more pronounced for pupae than for adult bees. In mites, DWV incidence was very high among the samples collected from the studied Palestinian districts. These results confirm the putative role of varroa in the transmission of this virus. This result is in agreement with those of Bowen- Walker et. al. (1999) who reported that varroa is highly effective vectors of DWV between bees under field conditions. In addition, other researchers of honeybee found that under natural and artificial varroa mite infestations, bee pupae contained significantly higher levels of DWV RNAs than mite- free pupae (Shen et. al. 2005b) . Interestingly, a large number of DWV- positive colonies were detected both for adult bees and for pupae or larvae throughout the year. According to Tentcheva et. al. (2004a) , DWV is thought to be responsible for wing deformities when infection occurs during the white- eyed pupation stage of bee development. However, DWV has been cited as potentially responsible for bee colony recession, but other studies have shown that this virus might be considered poorly pathogenic.

ABPV was the second most attacking virus to honeybees in the studied Palestinian territories. The virus was detected in varroa and its incidence in bee colonies was higher in the summer and in autumn. This suggests that the mite has a role in spreading this virus together with the possibility of virus transmission by contact between individual bees. SBV infection occupied the third highest rank as a virus attacking bee colonies in Palestine. The virus was found more frequently in adults than in pupae. Like ABPV, the virus has frequencies which were much higher during the summer and autumn. Similar SBV frequencies were previously found in healthy workers and pupae in Australia by an indirect SBV detection method (Anderson and Gibbs. 1988) . This latter work also showed that the occurrence of ABV was greater in the spring and in the summer. The seasonal variation



* Means with the same letter indicate no significant difference

Statistical analysis of the seasonal variation of DWV infecting both adult and immature bees revealed clear significant differences between seasons starting from spring until autumn. The maximum significant value of the virus incidence in both adult workers and broods was recorded during autumn in the studied districts. On the other hand, the maximum significance for the incidence of the other viruses attacking both adults and broods was mostly recorded during the summer season (Fig 2) .

In addition, data analysis based on the computed Z values revealed that the DWV was highly significant bee virus in the studied Palestinian territories (Fig 3) . Also, the virus occurrence was highly significant in varroa compared with other bee viruses (Fig 4) . No significant difference achieved when the DWV incidence is compared between Jenin and Tulkarm districts. Significant differences were found when the virus occurrence in these regions was compared with the virus outbreak in Nablus district.

ABPV infection of adult bee workers, the maximum values were found during summer to be 22%, 20% and 14% in Jenin, Tulkarm and Nablus respectively. In the case of pupa infection with ABPV, the highest recorded results were 12% in Jenin followed by 10% and 6% in Tulkarm and Nablus respectively. The maximum infection of bee workers with SBV was recorded in Jenin being 18% followed by 16% and 12% in Tulkarm and Nablus respectively. Brood infection with this virus achieved the maximum value of 9% in Jenin followed by 7% and 4% in Tulkarm and Nablus respectively (Fig 3).

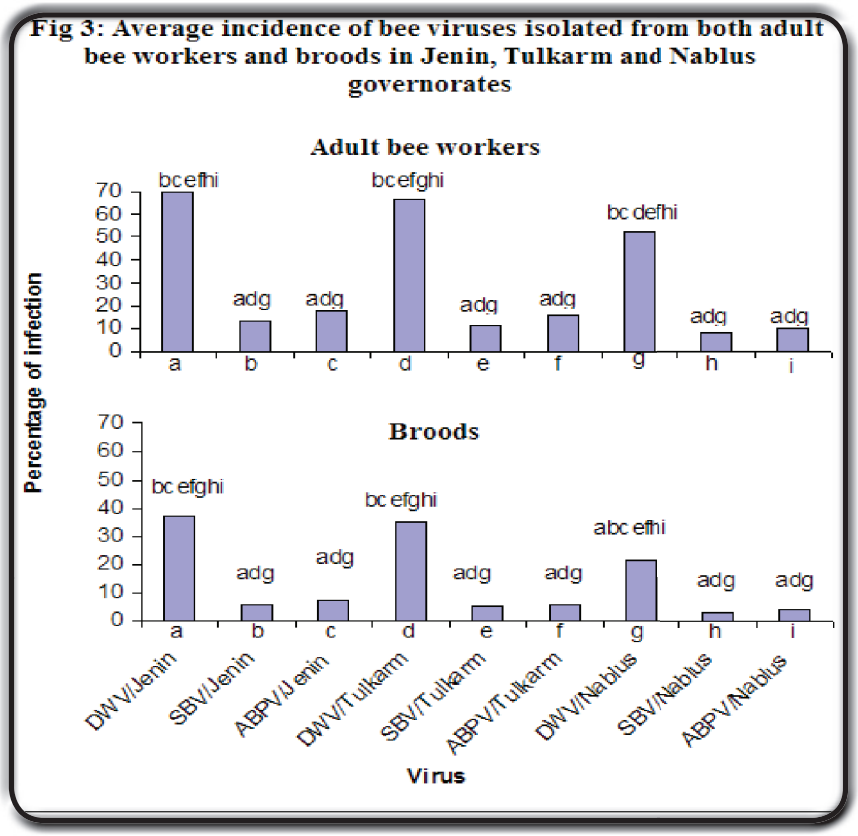
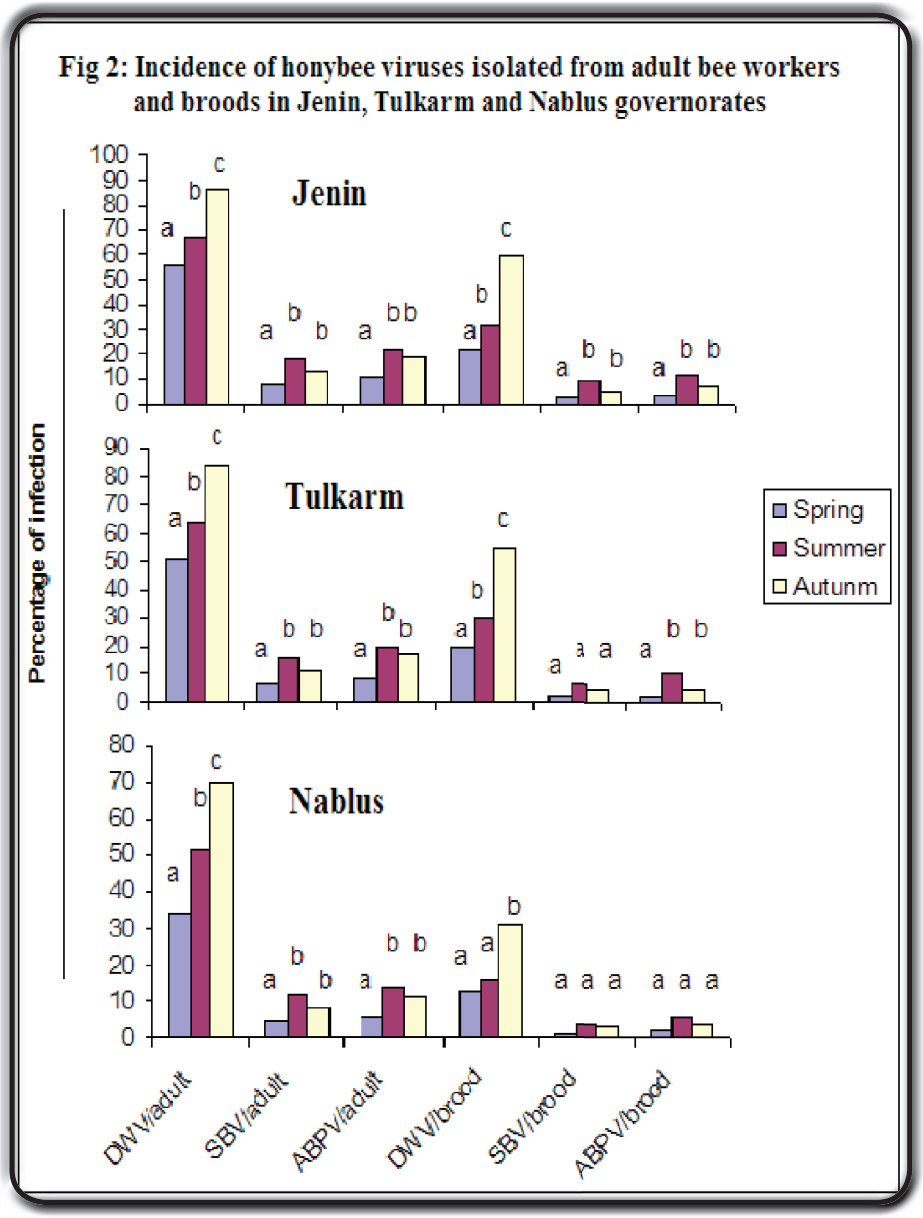


Fig 3: Average incidence of bee viruses isolated from both adult bee workers and broods in Jenin, Tulkarm and Nablus governorates

* Letters above the column indicate the treatments with significant difference

Based on PCR results, the studied viruses were detected in varroa. The prevalence of these viruses in the mite was slightly different, as follows: 93-95% for DWV, 2- 3% for ABPV, and 1- 2% for SBV. No significant differences were achieved between the virus prevalence in mite within the same location (Fig 4) .



* Means with the same letter indicate no significant difference.

The maximum occurrence of DWV in adult bee workers was recorded 86% during autumn in Jenin district followed by 84% and 70% for Tulkarm and Nablus respectively. With regard to bee pupae, the occurrence of the same virus was 59%, 55% and 21% for the same regions, respectively. For

Fig 1: RT- PCR detection of different honeybee viruses from infected honeybees. Different primers were used to amplify virus- specific sequences. Lane 1: 100 base pair DNA size marker. Lane 2 and 3: Deformed Wing Virus (DWV) . Lanes 4 and 5: Sac Brood Virus (SBV) . Lanes 6 and 7: Acute Bee Paralysis Virus (ABPV) . Lanes 8- 10: Negative control, RT- PCR without a template (lane: 8) and RT- PCR with a template from virus- free honeybees (lanes: 9 and 10) . For lanes 2- 7, even numbers represent viruses isolated from adult bee workers, while those with odd numbers represent viruses isolated from bee broods.

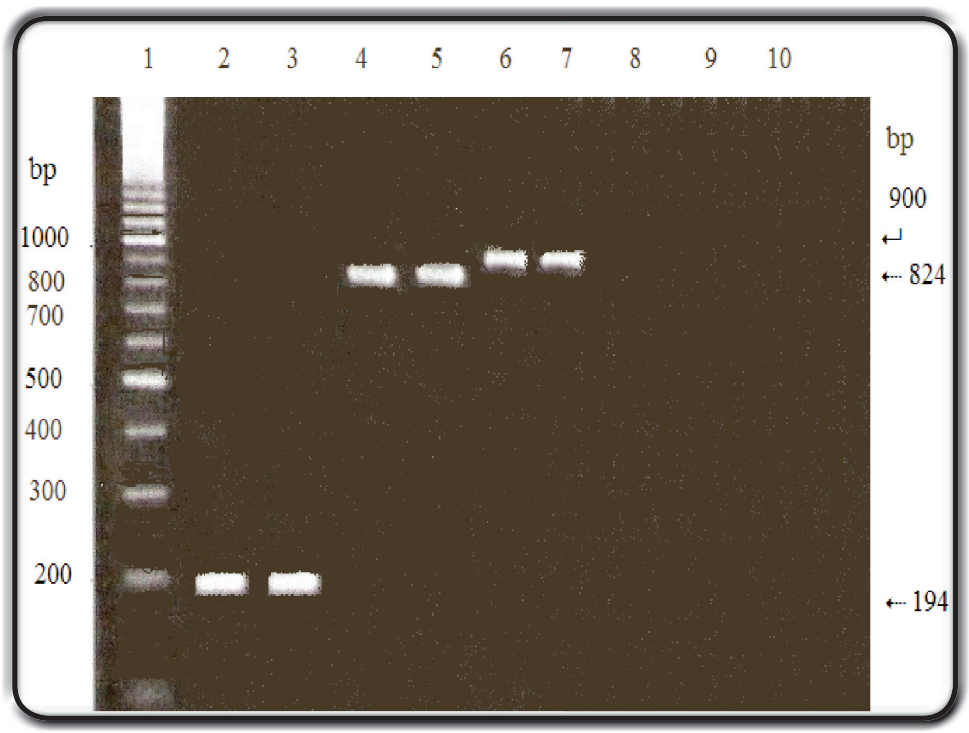
RT- PCR results showed seasonal variations in virus frequencies with both adults and pupae (Fig 2) . SBV was prominently found in the spring and summer in both adults and pupae. In adults, the SBV frequencies in colonies were 5- 8, 12- 18, and 8- 13% in the spring, summer, and autumn, respectively. In pupae, the values were 1- 2, 4- 9, and 3- 5%, respectively. The same distribution was observed for ABPV infections in adults, as the frequencies were 6- 11, 14- 22, and 11- 19% in the spring, summer, and autumn, respectively. In contrast, the DWV infections increased during the year both in adults and in pupae. The recorded DWV frequencies in the spring, summer, and autumn were 34- 56, 52- 67, and 70- 86%, respectively, for adults and 13- 22, 19- 31, and 21- 59%, respectively, for pupae (Fig 2) .

Statistical Analysis:

The survey and the sample collection from the studied regions were done according to the standards of the Completely Randomized Design (CRD) . The analysis of the data was conducted using the Two- Sample Tests of Proportions (TSTP) to compare the occurrence of the viruses in the studied regions. The results were then analysed using a level of significance when ά = 0.05 (Lind et. al. , 2005). Statistical analysis was done according to recommendation of Dr. Elias Dabeet, Department of Mathematics & Statistics, Faculty of Arts & Sciences, Arab American University of Jenin

Results:

In this study, twenty apiaries were selected from Jenin (7), Tulkarm (7) and Nablus (6). Ten hives were randomly identified in each apiary. Adult and immature bee samples were assayed for the presence of three honeybee viruses (DWV, ABPV and SBV) using RT- PCR. The results are shown in Figure 1.



were crushed in a sterile mortar in the presence of liquid nitrogen and then homogenized in 1 ml Trizol reagent (Biobasic). This was followed by chloroform extraction, isopropanol precipitation and centrifugation (16000 rpm). The pellets were washed twice with 70% ethanol, resuspended in nuclease- free water and stored at – 20°C.

RT- PCR Amplification:

Nuclic acid amplification was done in a 25- µl reaction mixture containing 1 X reaction buffer, 0. 2mM dNTP, 1 µM each of the two primers (Alltech Company, Paisley, UK.) for each virus, 2 mM MgS04, 0.1 U of avian myeloblastosis virus reverse transcriptase (Promega, USA) , 0.1 U of Tfl DNA polymerase (Promega) and 250 to 500 ng of total RNA (Haddad et. al. 2008, Tortora et. al 2004). The primer sequences and the expected amplicon size were shown in table 1:

Table 1:

The sequences of primer pairs used for detection of three honeybee viruses

Bee virus	Primer sequence (5 > - 3 >)	Amplicon size (bp)
DWV	F: CTTACTCTGCCGTCGCCCA	194
	R: CCGTTAGGAACTCATTATCGCG	
SBV	F: GCTGAGGTAGGATCTTTGCGT	824
	R: TCATCATCTTCACCATCCGA	
ABPV	F: TTATGTGTCCAGAGACTGTAT	900
	R: GCTCCTATTGCTCGGTTTTTC	

Reverse transcription at 48°C for 48 min. was followed by 40 cycles of 95°C for 30 s, 55°C for 1 min. and 68°C for 2 min. and a final extension at 68°C for 7 min. The amplified products were electrophoresed in 2% agarose gel, stained with ethedium bromide. Fragment size of the RT- PCR product was estimated depending on the standard curves of the typical relationship between the fragment size in base pair (bp) and the mobility of bands in 100 base pair DNA size marker (Sawalha, 2000). Negative controls were added in the experiment.

Bee Paralysis Virus (ABPV) , Black Queen Cell Virus (BQCV) , Sac Brood Virus (SBV) , Deformed Wing Virus (DWV) and Cloudy Wing Virus (CWV) , are very important to produce remarkable losses with recognizable clinical symptoms at certain life stages (Allen and Ball, 1996).

- Protecting honeybee colonies from diseases is a critical component of the beekeeping business. Recently, a large proportion of Palestinian beekeepers complained about considerable bee mortality in their hives, especially in Jenin district. Therefore, the current research aims to identify and investigate three honeybee viruses including DWV, ABPV and SBV using reverse transcription polymerase chain reaction (RT-PCR) to assist researchers and beekeepers to identity the diseases caused by these viruses, and to develop an appropriate disease control program to combat virus infections in honeybees. This is the first research that uses RT- PCR to study the honeybee viruses in Palestine

Materials and Methods:

Sample Collection:

Twenty apiaries with beekeepers willing to cooperate were selected, in Jenin (7) , Tulkarm (7) and Nablus (6) districts for random samples collection from both adult workers and broods. Ten hives were randomly identified in each apiary and samples were collected at different time intervals during 2008: in the spring (from March to 15 June) , summer (from 16 June to 15 August) , and autumn (from 16 August to November) . In the laboratory, the pupae were removed from their puparia using common pins, and the collected samples were frozen at –20°C until use. Varroa mites (100 mites per beehive) were collected at the end of August to the beginning of September following colony treatments with an acaricide (Amitraz) . Mite samples were stored as described previously (Shen. et. al. 2005a) .

RNA Extraction:

Total RNA extraction from bee viruses was performed as described by Haddad et. al. (2008). Individual frozen bee samples (one bee/ sample)

Introduction:

The beekeeping industry plays a key role in agricultural production in Palestine. The number of bee hives in Palestine is 65921; they are 48424 in West Bank and 17497 in Gaza. Jenin is the major Palestinian territory in beekeeping as it has 8298 bee hives followed by Tulkarm and Nablus districts with 5601 and 5134 bee hive, respectively. In the same sequence, the annual honey production in these territories is 65, 55 and 50 metric tons. These districts contribute for about 40% of beekeeping in the West Bank. (Palestinian Central Bureau of Statistics (PCBS), 2008). Beekeeping in Palestine is managed for honey production, secondary hive products (bee wax, pollen, royal jelly and propolis) , and as a source of bee stock (the sale of queens, "package" bees, and nucleus colonies) . Hobbyists (50 colonies or less) comprise the majority of beekeepers in the country. Sideliner (50- 500 colonies) and commercial (more than 500 colonies) beekeepers are not as numerous as the hobbyists, yet they provide most of the gross production of the bulk wholesale honey (PCBS, 2008) .

- Honeybees (Apis mellifera) are attacked by a wide variety of pathogens which are responsible for significant colony losses. Among honeybee pathogens, viruses pose one of the major threats to the health and well-being of honeybees and have caused serious problems for researchers and beekeepers (Allen and Ball. 1996, Nordstrom et. al., 1999, Tentcheva et al, 2004b; Todd et al.. 2007, Chen and Siede, 2007). Most adult honeybees carry symptomless viral infections (Anderson and Trueman. 2000, Shimanuki et. al. 1994). However, under conditions of stress caused by poor nutrition, inclement weather, or parasitism by varroa, viral populations can increase and cause symptoms in adult bees (Shimanuki, et. al. 1994).
- The honeybee is a host to at least 18 viruses that normally persist in the colonies as covert infections (Ball and Bailey, 1997) . Of these, Acute

Abstract:

Honeybee viruses cause a serious problem that beekeepers suffer persistently from, in Palestine. The presence of honeybee viruses is responsible, with other factors for the recurrent apiary collapse. In the current research, adult and immature bee samples collected from Jenin (1214) , Tulkarm (1016) and Nablus (1032) districts were tested for three honeybee viruses using RT- PCR and specific primers. Deformed Wing Virus (DWV) , Acute Bee Paralysis Virus (ABPV) , and Sac Brood Virus (SBV) were detected in the collected samples with different infection levels. The DWV was the predominant virus infecting honeybees in the studied Palestinian territories. Infection frequencies with this virus were 34 - 86% and 13 - 59% for adult bee workers and broods respectively. ABPV was the second most virus attacking bee colonies in the studied regions, followed by SBV. The maximum infections of adult bee workers with ABPV was 22% whereas 18% infection was recorded for SBV. The mixed infection with these viruses elucidates that they act together to cause apiary failure. This is the first report of the detection of bee viruses in Palestine using molecular techniques.

Key words: DWV, ABPV, SBV, Honeybee, Apis mellifera, RT- PCR.

ملخص:

تسبب فيروسات نحل العسل مشكلة خطيرة يعاني منها النحالون في فلسطين بشكل مستمر. لذلك، تعد هذه الفيروسات مع وجود العوامل الأخرى مسؤولة، وبشكل متكرر عن انهيار المناحل الفلسطينية. وقد جُمعت في هذا البحث عينات من النحل في أطوارها المكتملة وغير المكتملة من محافظات جنين وطولكرم ونابلس، ومن ثم فحصت لإمكانية إصابتها بثلاثة فيروسات باستخدام تفاعل البوليميريز المتسلسل ذي النسخ العكسي، والبادئات الوراثية. وقد بينت الفحوصات أن جميع العينات المجموعة من مناطق الدراسة كانت مصابة بنسب مختلفة بفيروس تشوه الأجنحة (DWV)، وفيروس الشلل الحاد (ABPV). وفيروس تكيس الحضنة (SBV). وكان فيروس تشوه الأجنحة هو الفيروس السائد في جميع المناطق الفلسطينية المدروسة، إذ تراوحت نسبة الإصابة به من ٣٤– ٨٦٪ للحشرات الكاملة، ومن ١٣– ٥٩٪ للحشرات في طور اليرقة. كذلك جاء فيروس الشلل الحاد في المرتبة الثانية من حيث مهاجمته، ومدى انتشاره في خلايا النحل،إذ وصلت أعلى إصابة لشغالات النحل بهذا الفيروس إلى ٢٢٪ في محافظة جنين. في حين ظهر فيروس تكيس الحضنة في المرتبة الثالثة من حيث الأهمية، إذ تراوحت نسبة إصابته لشغالات النحل من ٥– ١٨٪ في مناطق الدراسة. يعدُّ هذا البحث من البحوث البكر في فلسطين للكشف عن فيروسات النحل باستخدام التقنيات الجزيئية.

Detection of Three honeybee Viruses in Palestine using RT- PCR

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