

14<sup>th</sup> International Conference of Arab Beekeepers Union, 13-15 November 2023, Cairo-Egypt

## Prevalence of Viruses in Palestinian Honey Bee Colonies

By

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### ABSTRACT

Honey bees are critical for agricultural crops pollination. However, many countries have reported high annual colony losses caused by multiple possible factors. Diseases, including those caused by viruses of honey bees, are a significant cause of colony losses. However, nothing is known about the occurrence of viruses of honey bees in Palestinian honey bees. Therefore, eighty colonies from eight apiaries in Bethlehem and Hebron governorates were randomly selected and screened for the presence of 15 viruses of honey bees via quantitative PCR technique. Ten viruses were detected among which the Black Queen Cell virus (BQCV) was the most prevalent (96 % of all colonies) followed by Deformed Wing viruses (DWV) A (77%) and B (72%) and a new emerging virus, the Big Sioux River virus (BSRV, 70%). Other viruses (*Apis mellifera* filamentous virus (AmFV), Lake Sinai virus 1 (LSV-1), Acute Bee Paralysis virus (ABPV), Sacbrood virus (SBV), Israeli Acute Paralysis virus (IAPV), and Chronic Bee Paralysis virus (CBPV) had lower colony prevalences ranging between 9-29%. The study showed that most of the viruses were present in all apiaries, and multiple virus infections per colony were common in all colonies. Our findings fill a knowledge gap about the prevalence of viruses of honey bees in Palestine, which could assist in protecting the Palestinian beekeeping industry.

**Keywords:** Virus; honey bees; BSRV; prevalence; qPCR; Palestine

### 1. INTRODUCTION

The western honey bee (*Apis mellifera*) is one of the most valuable pollinators for many crops, wild flowering species, and the fruit industry (Klein *et al.*, 2007 and Potts *et al.*, 2016). However, the number of managed honey bee colonies decreased in many regions of the world, annual losses in USA, Middle East, and Europe accounted for 30%, 10-85%, and 1.8-53%, respectively

(Neumann and Carreck, 2010 and Bruckner *et al.*, 2023). These losses have been associated to parasites, pathogens, poor nutrition, sub-optimal beekeeping practices and pesticide exposure (Hristov *et al.*, 2021). Among the pathogens are viruses, which are widely recognised as a major cause of colony death (Chen and Siede 2007 and Beaurepaire *et al.*, 2020). More than 30 honey bee-associated viruses have been

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identified in the Western honey bee (*Apis mellifera*) (Chen and Siede, 2007; McMahon *et al.*, 2018 and Beaufrepaire *et al.*, 2020). Many of these bee viruses do not produce clear physical or behavioural symptoms in honey bees, while for others symptoms may exist but have not yet been identified. However, eleven of the viruses can severely impact honey bee health, *e.g.* deformed wing virus (DWV) type A and B, black queen cell virus (BQCV), chronic bee paralysis virus (SBPV), sacbrood virus (SBV), cloudy wing virus (CWW), and three closely related dicistroviruses belonging to the acute bee paralysis complex, namely Israeli acute paralysis virus (IAPV), acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) (Beaufrepaire *et al.*, 2020). Furthermore, a number of other viruses have recently been discovered in honey bees including Lake Sinai virus strain 1 (LSV1), Lake Sinai virus strain 2 (LSV2), big Sioux River virus (BSRV), Aphid lethal paralysis virus (ALPV) and Bee Macula-Like Virus (Runckel *et al.*, 2011 and de Miranda *et al.*, 2015). Their pathogenicity for honey bees including the effects on the colony level are currently under examination.

DWV is the most common and detrimental virus in the western honey bee (Chen and Siede 2007; McMahon *et al.*, 2018 and Beaufrepaire *et al.*, 2020). Infected adult bees show typical symptoms like crumpled or aborted wings, malformed bodies especially shortened abdomens and severely shortened adult life span for emerging workers (McMahon *et al.*, 2018).

Three variants were identified. These are, type A (DWV-A), which has been attributed to the global decline in honey bees (Kevill *et al.*, 2019), type B (DWV-B), originally named *Varroa destructor* virus-1 (VDV-1), which has rapidly dispersed worldwide since its first description in 2004, is potentially replacing DWV-A (Paxton *et al.*, 2022) and is associated with the overwintering bee mortality (Natsopoulou *et al.*, 2017) and type C (DWV-C) (Mordecai

*et al.*, 2016) has been discovered recently. Its impact of on honey bees is unknown yet.

BQCV is one of the most prevalent and widespread viruses in honey bee colonies (Ding *et al.*, 2016, D'Alvise *et al.*, 2019; Beaufrepaire *et al.*, 2020 and Mayack and Hakanoğlu 2022). It can kill queen larvae and pupae and may also kill the adult bee workers (Al Nagggar and Paxton, 2020).

The closely related dicistroviruses ABPV, IAPV, and KBV generally persist at a low level of infection within the colony. However, they are extremely virulent when injected into pupae or adults (McMahon *et al.*, 2018). If ABPV is transmitted by *V. destructor*, it causes immobility of infected adult bees and their death in short time (Martin *et al.*, 2012).

SBV is also a common and globe-prevalent virus of honey bees (Chen and Siede 2007 and Beaufrepaire *et al.*, 2020). It is transmitted to larvae by SBV-infected nurse bees (McMahon *et al.*, 2018). Infected queen larvae fail to pupate acquiring a sac-like appearance and finally die. SBV can also infect adult bees without clinical signs of disease, but these bees may have a shorter life span (Bailey and Fernando, 1972).

Natural transmission of infectious viruses to honey bees occurs mainly horizontally among bees of the same generation, including via oral/and/or body contact (Yañez *et al.*, 2020) and vertical virus transmission between generations as well as venereal transmission, where virus is transmitted from drones to queens during the mating flights, also occur (Yañez *et al.*, 2012). The emergence of *V. destructor* as an example of vector-mediated virus transmission has also significantly altered the viral landscape in honey bee populations by increasing virus transmission and causing selection of more virulent virus strains (Genersch and Aubert, 2010 and McMahon *et al.*, 2016). Especially ABPV, KBV, CBPV, DWV A and B are transmitted by *Varroa* (reviewed by Beaufrepaire *et al.*, 2020). Although numerous studies have

been conducted to assess the prevalence of viruses in honey bee colonies around the world (Beaurepaire *et al.*, 2020) data about the occurrence and prevalence of viruses of honey bees in Palestine are unknown. The current study aims to determine the prevalence of 15 common bee viruses in honey bee colonies in Palestine.

## **2. MATERIAL AND METHODS**

### **2.1. Honey bee samples**

To investigate the prevalence of viruses of honey bees in Palestine, eight apiaries in two governorates (Hebron and Bethlehem) were targeted, and ten randomly selected colonies per apiary were analyzed. In total, 80 colonies were visited in the spring of 2017 and sampled. Around 100 adult honey bees per colony were collected from peripheral frames of the hive and kept at -25°C in Hebron University-Palestine. Then, samples were transferred to Germany and stored at -80°C until processing.

### **2.2. Varroa mite infestation rate**

All stored bee samples were checked for *Varroa* mite numbers using Wild M5A stereomicroscope at 50x magnification. The *Varroa* mite infestation level per colony was calculated by the equation below:

$$\text{infestation level [\%]} = \frac{\text{number of varroa counted}}{\text{number of examined bees}} \times 100$$

### **2.3. RNA extraction and virus detection**

Pools of 30 bees per colony were taken for total RNA extraction, crushed in plastic RNase-free mesh extraction universal bags (BioReba, Reinach, Switzerland) with 10 ml of diethylpyrocarbonate (DEPC)-treated distilled water using an automated bee grinder (Homex6, BioReba, Reinach, Switzerland) after snap freezing on dry ice. 100 µl of the homogenate were mixed with 400 µl of RLT-buffer containing 1% β-mercapto ethanol and RNA was extracted via RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA concentration was

determined using an Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Germany). A total of 1 µg of RNA was used for cDNA synthesis using oligo(dT)<sub>18</sub> primers (Thermo Scientific) and reverse transcriptase (M-MLV and Revertase, Promega, Mannheim, Germany) following the manufacturer's instructions.

The resultant cDNA was diluted 1:10 prior to its use in quantitative real-time PCR (qPCR) which was performed on a Bio-Rad CFX thermal cycler (Bio-Rad, Feldkirchen, Germany) using SYBR green Sensimix (Bioline, Luckenwalde, Germany) and the specific primers listed in supplementary data file Table S1 to determine viruses' prevalence of fifteen common bee RNA viruses: DWV-A, DWV-B, BQCV, CBPV, ABPV, SBV, SBPV, KBV, IAPV, BSRV, AmFV, LSV-1, LSV-2, ALPV, and BeeMLV. qPCR amplification program steps included: 5 min at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at 57°C, and 30 s at 72°C, followed by 5 min at 72 °C. Each sample was carried out in duplicate. Quality control checks were run on each qPCR 96-well reaction plate. To check that the correct template had been amplified, PCR products were denatured for one minute at 95°C, cooled to 50°C for one minute, and then a melting profile was generated from 50°C to 95°C (increment 0.5°C/second). qPCRs were repeated for samples whose melting profiles suggested non-specific amplification (f.i. broad peak) or an incorrect melting temperature. qPCRs were also repeated for samples whose duplicate technical Cq values differed by more than 0,5. In addition, a virus-free (negative control) and a virus-infected (positive control) sample were included on each 96-well plate. *Apis mellifera* rp49 gene (Lourenço *et al.*, 2008) was also amplified for all samples as a honey bee internal reference marker to confirm that RNA extraction and cDNA synthesis were successful.

## 2.4. Statistical analysis

Statistics were performed using R 4.2.1. (R Core Team, 2022) Significance levels were set to  $p\text{-value} \leq 0.05$  for all statistical tests, and  $p\text{-values}$  were adjusted for multiple testing using Bonferroni-Holm-correction. Mixed effect models were implemented using the lmer function within the lme4 R package (Bates *et al.*, 2015). Post-hoc tests for differences in prevalences of viruses were performed with Tukey tests using the command 'glht' (package 'multcomp'). The correlations between virus prevalences or between viruses and varroa presence were analyzed by Pearson correlations using R package cor mat 'rstatix'. Log-transformed data of the virus loads determined by qPCR were used to compare the differences between varroa infected colonies and non-varroa infected colonies.

## 3. RESULTS AND DISCUSSION

Ten out of fifteen viruses were successfully detected in Palestinian honey bee colonies by qPCR: ABPV, AmFV, BQCV, BSRV, CBPV, DWV-A, DWV-B, IAPV, LSV-1, and SBV, while five viruses ALPV, Bee MLV, LSV-2, KBV, and SBPV were not detected in any of the sampled colonies.

BQCV (96%) was the most prevalent virus among the detected ten honey bee viruses followed by DWV-A (77%), DWV-B (72%) and BSRV (70%) (Fig.1). The other viruses occurred to significantly lesser extent in descending order: AmFV (29%), LSV-1 (19%), ABPV (10%), SBV (10%), CBPV (9%) and IAPV (9%) (Fig.1; LMM; Tukey post-hoc test:  $p < 0.05$  for multi-variable comparisons between viruses, see supplementary file, (Table S2).

Viral infections and co-occurrence varied between apiaries. BQCV, DWV-A, DWV-B, AmFV, and BSRV were detected at all apiaries (Table 1). ABPV and LSV1 were

detected in 50% of the apiaries whereas CBPV, IAPV, and SBV were only detected in 20% of the apiaries (Table 1). Moreover, the number of detected viruses were varied between colonies. Multiple virus infections were detected in all targeted colonies, while not a single colony was infected by only one virus (Fig. 2). The average of multiple infections amounted to  $3.9 \pm 1.1$  viruses/colony (mean  $\pm$  SD).

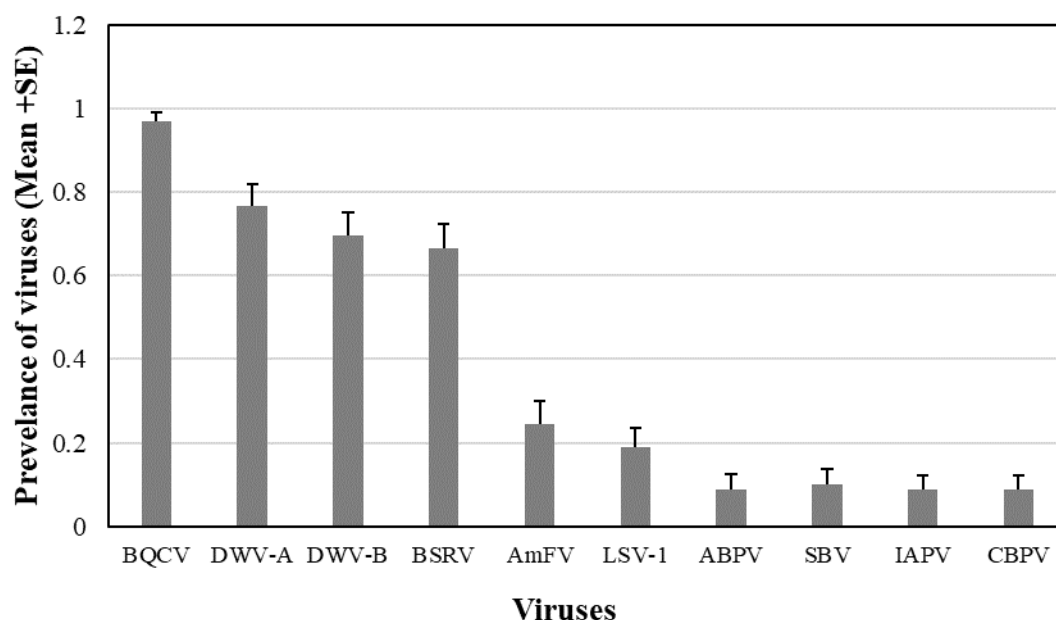
Additionally, colony co-occurrence relationships between viruses and varroa infestation were performed using correlation analysis (Fig. 3). It revealed only a strong positive correlation between the RNA viruses IAPV and ABPV ( $r=0.75$ ,  $p < 0.05$ ), a weak positive correlation between CBPV and SBV ( $r=0.32$ ), SBV and LSV1 ( $r=0.30$ ), DWV-B and AmFV (0.29), a weak negative correlation between IAPV and BSRV ( $r=-0.26$ ), and a weak negative correlation between varroa mite and BSRV (-0.26). Other significant correlations were not detectable mainly due to the low sample size and the low *Varroa* infestation levels ( $0.3 \pm 0.1$  mites/100 bees). However, viral loads of DWV-A were 50 times and of DWV-B 1000 times higher in *Varroa*-infested colonies than in colonies without *Varroa* as determined by qPCR (Students t-test, log transformed data,  $p < 0.05$  for both viruses,  $n_{\text{Varroa}}=8$ ,  $n_{\text{w/o Varroa}}=45$  and 40, resp). For other *Varroa*-transmitted viruses (ABPV, KBV, CBPV) no relation between virus loads and *Varroa* infestation were detected (data not shown).

The current study had been conducted to assess the prevalence of viruses of honey bees in Palestine for the first time. Out of 15 studied honey bee viruses ten viruses were detected. BeeMLV, KBV, LSV-2, ALPV, and SBPV could not be found in any of the colony samples. Four of the ten were the most prevalent: BQCV, followed by DWV-A, DWV-B, and BSRV, which were detected in all apiaries and most colonies.

**Table (1): Prevalence of honey bee viruses in different apiaries.**

Location	Region	Virus Prevalence (%)										No. Infected
		BQCV	DWV-A	DWV-B	BSRV	AmFV	LSV1	ABPV	SBV	IAPV	CBPV	
Hindaza	B	100	70	60	100	10	0	10	0	0	0	6
Shawawra	B	100	89	100	44	33	0	0	11	0	0	6
Wadi-Fukin	B	100	88	88	88	25	50	25	0	0	13	8
Ubeidyia	B	91	82	100	27	36	0	0	55	45	0	7
Battir	B	100	56	44	89	22	44	0	0	11	0	7
Majd-Dura	H	100	90	40	60	40	40	30	0	0	50	8
Al-Dahyiria	H	80	100	60	60	40	20	20	0	0	0	7
Bayt-Ula	H	86	43	86	100	29	0	0	0	0	0	5

1. Region B: Bethlehem, H: Hebron.
2. n=10 colonies per apiary
3. ABPV: acute bee paralysis virus, BQCV: black queen cell virus, CBPV: Chronic bee paralysis virus, DWV-A: deformed wing virus-A, DWV-B: deformed wing virus-B, IAPV: Israeli acute paralysis virus, SBV: sacbrood virus, BSRV: Big Sioux Rver virus, AmFV: *Apis mellifera* filamentous virus, LSV1: Lake Sinai Virus-1.



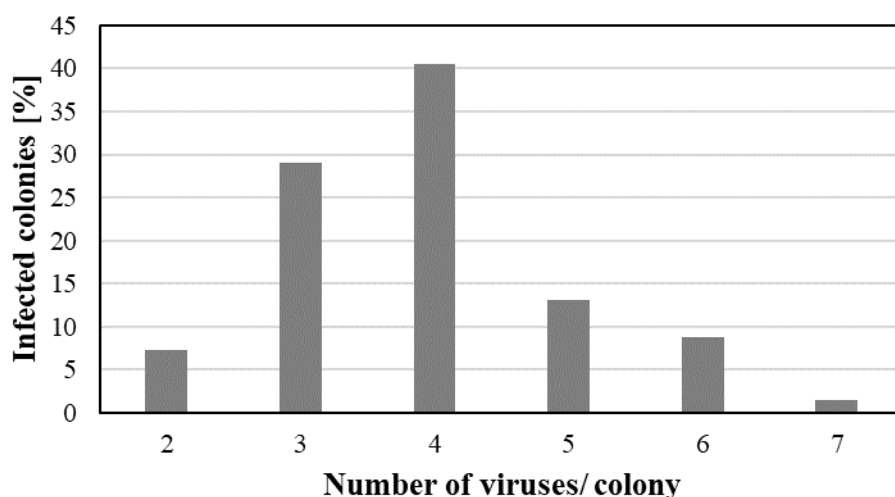
**Fig (1): Prevalence of viruses in Palestinian honey bee colonies.**

Columns represent the mean prevalence/presence of viruses (±SEM). Different letters represent significant differences between the prevalences of the viruses of honey bee. (LMM; Tukey post-hoc test:  $p < 0.05$ ;  $n = 80$  tested colonies).

BQCV was the most prevalent virus detected in the inspected samples. This result corresponds to its high prevalence and occurrence worldwide (Beaurepaire *et al.*, 2020). Studies in neighboring countries found a prevalence of 29% in Syria (Abou Kubaa *et al.*, 2018), 70% in Egypt (Kandel *et al.*, 2023) and 100% in Turkey (Mayack and Hakanoğlu, 2022). BQCV is currently considered a benign viral pathogen of adult

honey bees, possibly because its mode of horizontal transmission is primarily direct by food exchange among nestmates which does promote high virus replication (Al Naggar and Paxton, 2020). If BQCV transmission becomes vector-mediated, it may elicit adverse health effects.

The second most prevalent viruses were the *Varroa* mite transmitted deformed wing virus variants DWV-A (77%) and DWV-B



**Fig. (2): Occurrence of multiple virus infections in honey bee colonies.**

Multiple viruses were detected in all sampled colonies. n=80. Mean  $\pm$ SD = 3.9  $\pm$ 1.1 viruses/colony.

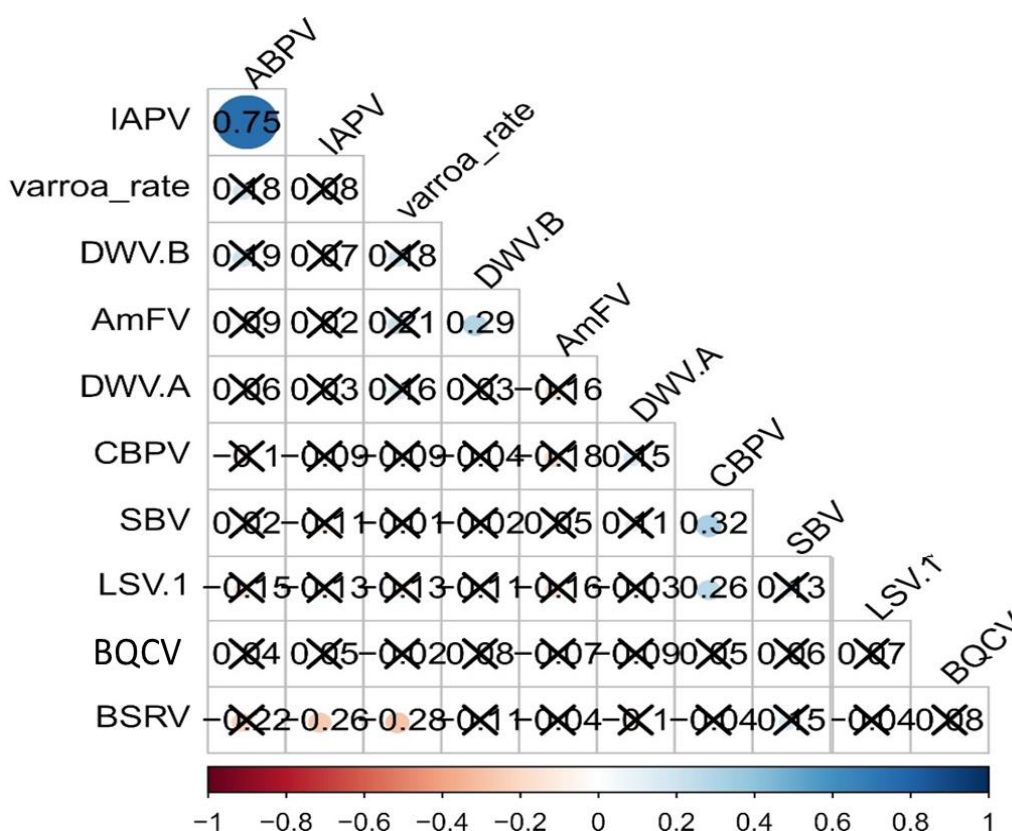
(72%). DWV-B showed similar high prevalence as in Europe (96%) (Natsopoulou *et al.*, 2017 and McMahon *et al.* 2018) and USA (66%) (Ryabov *et al.*, 2017). But in Egypt, DWV-B was not detected (Kandel *et al.*, 2023). The high DWV prevalence in Palestine could be explained by the virus spread through oral, faecal and/or environmental transmission (Beaurepaire *et al.*, 2020 and De Miranda *et al.*, 2013) or by transmission through *Varroa* mites (McMahon *et al.*, 2018), as reflected by several orders of magnitude higher virus loads of DWV A and B in *Varroa* infected colonies compared to those of non-*Varroa* infected colonies.

BSRV (70%) was also one of the most prevalent viruses in the samples. This result is quite surprising, since this virus had been rarely detected in honey bees. It was initially discovered in honey bees by specific PCR in 28 of 197 time-course samples in Mississippi and South Dakota (USA) mainly in July (Runckel *et al.*, 2011). As yet a second study from Turkey only reported minute amounts of virus RNA in worker bees by RNA-seq reads (Mayack and Hakanoğlu, 2022). The biology of the virus and its potential pathogenicity and

transmission is not yet known, BSRV appears to be a multi-host pathogen having been also detected by metagenomics analyses in mosquitoes and soybean aphids and maize (Feng *et al.*, 2017, Shi *et al.*, 2015 and Wamonje *et al.*, 2017).

Other viruses occurred with prevalences between 29% (AmFV), 19% (LSV-1), and around 10% (ABPV, IAPV, SBV, and CBPV). Their clinical relevance for the honey bee host were not analysed and epidemiological sample sizes were too small for scientific conclusions. Other studies provided strong correlative evidence for the members of the ABPV/KBV/IAPV clade and for DWV A and B being involved in colony losses (Cornman *et al.*, 2012; Cox-Foster *et al.*, 2007 and Genersch *et al.*, 2010).

Moreover, the current study revealed multiple concurrent infections of honey bee viruses with an average of 4 viruses per colony that varied subtly across locations. The results are consistent with previous research that has also reported simultaneous multiple infections of honey bee viruses (D'Alvise *et al.*, 2019; Ding *et al.*, 2016 and Kandel *et al.*, 2023).



**Fig. (3): Co-occurrence of viruses in honey bee colonies.**

Correlation matrix analysis was performed by using R-package: cor\_mat (rstatix), Pearson test, p<0.05.

In conclusion, this is the first comprehensive study on the prevalence of viruses in Palestinian honey bee colonies, revealing that BQCV, DWV-A and B, and BSRV are widely distributed. The data could assist in the protection of Palestine’s beekeeping industry and fill a knowledge gap of the global honey bee virome. Future studies are needed to understand the impact of the detected viruses on honey bee health.

**ACKNOWLEDGEMENT**

We thank the German Ministry of Education and Research (BMBF) and Palestinian Ministry of Higher Education (MOHE) Project No. PalGer2015-34-053 for funding this research, the Katholische Ausländer Dienst (KAAD), Germany, granting the scholarship for Mohammad and the beekeepers for their cooperation and providing honey bee colonies.

**Authors’ contributions**

All authors contributed in conceptualization, methodology, software, validation, formal analysis investigation, resources, data curtain, writing the original draft preparation, writing, review, editing, supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript.

**Competing interests**

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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## انتشار الفيروسات في مستعمرات نحل العسل الفلسطيني

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### ملخص

يعتبر نحل العسل مهم جداً لتلقيح المحاصيل الزراعية. إلا أن العديد من البلدان أبلغت عن خسائر سنوية عالية في خلايا نحل العسل بسبب عوامل متعددة كثيرة. تعتبر الأمراض التي تصيب نحل العسل ومنها الفيروسات من أسباب الخسائر الكبيرة لمستعمرات نحل العسل. ومع ذلك، لا يوجد معلومات عن انتشار فيروسات النحل في مستعمرات نحل العسل في فلسطين. لذلك، تم اختيار ثمانين مستعمرة نحل من ثمانية مناحل في محافظة بيت لحم والخليل بشكل عشوائي وفحصها ومسحها بحثاً عن انتشار 15 فيروساً من نحل العسل باستخدام تقنية تفاعل البوليميريز المتسلسل الكمي. تم الكشف عن عشرة فيروسات من بينها فيروس خلية الملكة السوداء (BQCV) الذي كان الأكثر انتشاراً (96% من جميع المستعمرات) تليها فيروسات تشوه الاجنحة (77%) و ب (72%) وفيروس جديد ناشئ هو فيروس نهر سيوكس الكبير (BSRV)، (70%). وقد كانت الفيروسات الأخرى (AmFV و IAPV, SBV, ABPV, LSV-1) أقل انتشاراً وتتراوح بين 9-29%. أوضحت الدراسة أن معظم الفيروسات كانت موجودة في جميع المناحل وكانت الإصابات الفيروسية المتعددة لكل خلية نحل شائعة في جميع المستعمرات. النتائج التي توصلنا إليها تسد الفجوة المعرفية حول انتشار فيروسات نحل العسل في فلسطين، ويمكن أن تساعد في حماية صناعة تربية نحل العسل الفلسطينية.

المؤتمر الرابع عشر لإتحاد النحالين العرب، 13-15 نوفمبر (2023)، القاهرة- مصر  
المجلة المصرية للعلوم الزراعية (2024) المجلد 75 (إصدار خاص): 32-41.