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Can the examination of different types of hive samples be a non-invasive method for detection and quantification of viruses in honey bee (*Apis mellifera* L.) colonies?

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Abstract

Introduction: Honey bee viruses have been shown to negatively affect the vigour and longevity of European honey bees (*Apis mellifera* L). In the present work, beehive materials were tested for their potential to serve as non-invasive samples for honey bee virus detection. **Material and Methods:** Honey, pollen, hive debris, hive grid smears and forager honey bees were collected from 24 hives at four locations in the Czech Republic. Deformed wing virus (DWV), acute bee paralysis virus (ABPV), sacbrood virus (SBV) and black queen cell virus (BQCV) were detected using a reverse transcription PCR (RT-PCR) and real-time quantitative RT-PCR and the results for bees and alternative materials compared. **Results:** All forager bee samples contained DWV, BQCV and SBV and 54.2% had ABPV. When comparing beehive materials to bees, the most promising results were obtained from honey and pollen samples, with BQCV and SBV detected in all honey samples and ABPV in 12.5%. Detection of SBV was achieved in 91.6% of pollen samples, detection of BQCV in 87.5% and detection of DWW in 75%. The results for debris and smears were less consistent with the viral profile of the forager samples. **Conclusion:** The best candidate materials for honey bee virus detection in a non-invasive technique are honey and pollen.

Keywords: acute bee paralysis virus, black queen cell virus, deformed wing virus, honey bee viruses, sacbrood virus.

Introduction

The honey bee, an important, globally widespread insect pollinator, plays an important role in natural ecosystems, and as such, represents a key component in biodiversity preservation. The European honey bee (*Apis mellifera* L.), in particular, is extremely important commercially as a pollinator of economic crops. Honey bees are increasingly threatened by a large number of pathogens and parasites, including several clinically significant bee viruses that are commonly present at low viral loads with no obvious signs of infection. In recent years, however, the global spread of an ectoparasitic mite, *Varroa destructor*, has led to an increase in the clinical manifestations of such viral diseases (13) and is one of the factors contributing to colony collapse disorder, which has caused major losses of honey bee workers (29).

Previous studies have confirmed the presence of viruses in pollen collected by honey bees, including clinically important viruses such as deformed wing virus (DWV), Israeli acute paralysis virus, chronic bee paralysis virus, black queen cell virus (BQCV), acute bee paralysis virus (ABPV), Kashmir bee virus (KBV) and sacbrood virus (SBV) (3, 4, 16, 19, 27, 28, 30). Furthermore, KBV and SBV have been detected in different bee food sources, such as honey and royal jelly (25), while DWV has been detected in larval food (30). High loads of such viruses in pollen and honey have been associated with increased bee mortality (23).

As a eusocial species, honey bees are highly prone to pathogen transmission, the large number of

individuals and strongly organised social structure of the colony and the resultant close physical contact leading to rapid pathogen transmission. The bees may be infected directly through contaminated food or indirectly by contact with excreted faeces (3), the faecal–oral transmission route being considered the most common route of virus transmission (11). Studies have also shown that a virus can enter the hive following contact between a forager bee and a contaminated plant, potentially leading to inter-species transmission of viruses (11, 16, 27). Furthermore, the presence of a virus in bee food would bring about rapid infection of the honey bee colony with subsequent contamination of hive structures through the bee's work activity. Viral transmission *via* contaminated food would suggest that honey and/or pollen could be used as a non-invasive tool for monitoring the honey bee viral burden within the hive. In the present study, a comparison is made of the burdens of a range of honey bee viruses in bees and different types of material originating from the bees' hive that could potentially be used for non-invasive monitoring.

Material and Methods

Samples were collected during May and June 2021 from 24 different beehives at four different locations in southern Moravia, Czech Republic (Fig. 1). Five sample types were collected from each beehive, *i.e.* bees and honey, pollen, hive debris and hive grid smears. Fifty apparently healthy forager bees were collected at the entrance of each beehive and transported to the laboratory alive, where they were subsequently frozen at −20°C. Smears from the iron grid of the hive bottom board were obtained using sterile swabs dipped in physiological saline solution. A pooled honey sample was obtained from three honeycombs in each hive using a sterile 50 mL test tube, the edge of the open test tube being gently pressed against open honey cells and the honey being allowed to flow down the tube. Likewise, a pooled fermented pollen sample was taken from the cells of three different combs in each hive using tweezers. Samples of hive debris were collected from diagnostic pads inserted under the grid of the bottom board. All samples were frozen and stored at −80°C until further analysis.

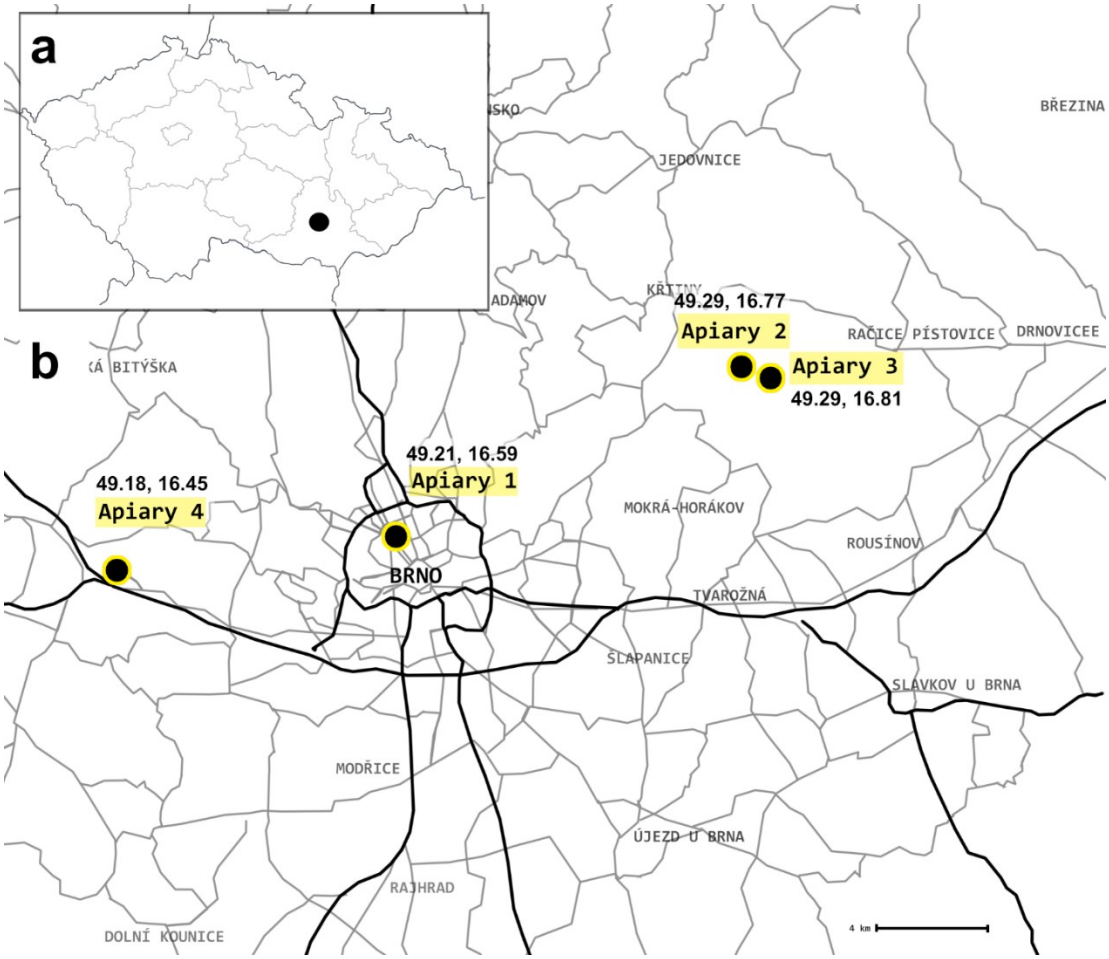

Fig. 1. Map indicating the sample collection area within the Czech Republic (a) and locations of the four sample collection sites (Apiaries 1–4) along with their respective GPS locations (b)

Extraction of nucleic acids. Prior to nucleic acid extraction, the swabs from the bottom board were homogenised in 1 mL of TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) in a homogeniser with 2.8 mm ceramic beads (OMNI International, Kennesaw, GA, USA) set at 3,500 rpm for 2 min. Likewise, pooled samples of the bees from one hive (each n = 50) were homogenised in 30 mL of TRI Reagent. A 100 µL aliquot of the homogenate, which approximately corresponded to the amount obtained from one bee weighing 100 mg, was used in the extraction process. For the debris, pollen and honey samples, the homogenisation process was omitted and 1 mL of TRI reagent was added directly to 100 mg of each material. Subsequently, the homogenates, debris, pollen and honey samples were centrifuged for 15 min at 22,000 rpm and the supernatant used for extraction of viral nucleic acids.

Extraction of nucleic acids from debris was carried out according to the certified methodology of Prodělalová *et al.* (21). Based on previous experience working with similar materials, the nucleic acids extracted from debris samples were first purified on magnetic beads using the Chemagic Viral DNA/RNA Kit (Perkin-Elmer, Waltham, MA, USA) and following the manufacturer's instructions, in order to remove enzyme inhibitors, which could potentially affect the success of nucleic acid replication. The nucleic acids thus obtained were stored at −80°C until further analysis.

Molecular detection of viruses. Once extracted, the nucleic acids were subjected to quantitative PCR (qPCR) using the Luna Universal One-Step RT-qPCR Kit (New England BioLabs, Ipswich, MA, USA) for molecular detection of the three viruses DWV, BQCV and SBV, each sample being screened twice to rule out false positives. The reaction was carried out in accordance with the standard protocol provided by the manufacturer, with 40 amplification cycles used for each run. The amount of each component was slightly modified to achieve a total volume of 15 µL per reaction (3.75 µL of RNA). The qPCR results were then processed using the Lightcycler 480 software (Roche Diagnostics, Basel, Switzerland). The absolute quantification/2nd derivative maximum algorithm was employed to calculate the concentration of viral load based on standard viral curves. Two pairs of primers targeting two different genomic regions (helicase-coding and RNA-dependent RNA polymerase–coding) were used for real-time detection of DWV to ensure increased screening effectivity (Table 1). Owing to a lack of standards for the helicase region of DWV, viral loads were only calculated for the DWV-positive samples obtained in the reaction with RNA-dependent RNA polymerase–targeting primers.

The samples were additionally screened for the presence of ABPV using a standard PCR as the qPCR method for the detection of ABPV was not available at the time of testing. First, cDNA was synthesised using the Protoscript II First Strand cDNA Synthesis Kit (New England BioLabs), following which it was subjected to a PCR reaction using OneTaq Hot Start Quick-Load 2X Master Mix with standard buffer reagent (New England BioLabs) following the standard protocol provided by the manufacturer (for the primers used in the reaction) (Table 1). The PCR products then underwent electrophoresis on 1.5% agarose gel, the products being visualised by transillumination. A standard PCR reaction screened for BQCV, using a different pair of primers (Table 1).

Statistical analysis. The frequency of PCR-positive and -negative virus samples for each location matrix were compared using Pearson's chi-squared test. In cases where the frequency did not meet the conditions of good approximation, we used Fisher's exact test or the chi-squared test with Yates correction. The results of qPCR viral load quantification were first tested for normality using the Shapiro–Wilk test. As the data proved to be non-normally distributed, the non-parametric Kruskal–Wallis analysis of variance test with subsequent multiple comparisons to compare the copies/µL of analyte in individual matrices for each virus tested was used.

Table 1. Primers and probes used in the qPCR and PCR reactions

Target virus	Method	Primer/probe	Target region	Reference
DWV	qPCR	qDWV-1 F,R	Helicase	2
		qDWV-1 P		21
		qDWV-2 F,R,P	RdRP	12
BQCV	qPCR	qBQCV F,R	7834–8119 bp	15
		qBQCV P		20
	PCR	BQCV1 F,R	379–700 bp	14
SBV	qPCR	qSBV F,R,P	5´NTR	20
ABPV	PCR	ABPV-1 F,R	RdRP	26
		ABPV -2 F,R	34–240 bp	14

bp – base pairs; DWV – deformed wing virus; BQCV – black queen cell virus; SBV – sacbrood virus; ABPV – acute bee paralysis virus; F – forward; R – reverse; P – probe

The Mann–Whitney U test was then used to compare the number of copies in pollen and bee DWV-positive samples. All statistical analyses were performed using UNISTAT v. 6.0.07 (Unistat, London, UK) or Statistica for Windows (TIBCO, Palo Alto, CA, USA), with the level of significance in each case set at $P < 0.05$.

Results

All of the selected viruses except ABPV were detected in all pooled bee samples (Table 2). Deformed wing virus was present at relatively low concentrations (1.13×10^3–1.31×10^3 viral copies/µL of analyte) in all samples except one, where the concentration reached 1.86×10^8 copies/µL. In contrast, the concentrations of BQCV and SBV were substantially higher, with BQCV concentrations relatively consistent at between 10^6 and 10^8 copies/µL and concentrations of SBV more variable at between 10^4 and 10^9 copies/µL (Tables 4 and 5). The presence of ABPV was confirmed in 54.2% of bee samples, representing all samples collected from Apiary 4, levels in bees from Apiaries 1–3 being below the level of detection (Table 3). As with the bee samples, BQCV and SBV were detected in all honey samples, while DWV was recorded in 15 (62.5%) samples and ABPV in just 3 (12.5%) samples (Tables 2 and 3). Viral loads in honey analytes ranged from 10^4 to 10^6 for both BQCV and SBV, one analyte having a concentration of 10^7 BQCV particles (Table 5). Unfortunately, the viral loads of DWV in honey could not be calculated as viral standards for the primers targeting the helicase region were unavailable. Sacbrood virus was detected in 22 (91.7%) of the pollen samples, BQCV in 21 (87.5%) and DWV in 18 (75%).

Table 2. Results of virus detection in bees, pollen, honey, hive debris and hive grid smears, using an end-point PCR (black queen cell virus (BQCV)) and a qPCR (deformed wing virus (DWV), black queen cell virus (BQCV) and sacbrood virus (SBV))

	DWV					BQCV					SBV				
Sample	Bees	Pollen	Honey	Debris	Smears	Bees	Pollen	Honey	Debris	Smears	Bees	Pollen	Honey	Debris	Smears
A1 1	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−
A1 2	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−
A1 3	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−
A1 4	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−
A2 1	+	+	−	−	−	+	−	+	−	+	+	−	+	+	+
A2 2	+	−	−	−	−	+	+	+	−	−	+	+	+	+	−
A2 3	+	+	−	+	−	+	+	+	+	−	+	+	+	+	+
A2 4	+	−	−	−	−	+	+	+	−	+	+	+	+	+	+
A2 5	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
A2 6	+	−	+	+	−	+	−	+	+	+	+	+	+	+	+
A3 1	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
A4 1	+	+	+	−	−	+	+	+	−	−	+	+	+	−	−
A4 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A4 3	+	+	−	−	−	+	+	+	−	−	+	+	+	+	−
A4 4	+	+	−	+	−	+	+	+	−	−	+	+	+	+	−
A4 5	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
A4 6	+	−	+	−	−	+	−	+	−	+	+	−	+	+	−
A4 7	+	+	+	−	−	+	+	+	−	−	+	+	+	−	−
A4 8	+	+	−	−	−	+	+	+	−	+	+	+	+	−	+
A4 9	+	+	−	−	−	+	+	+	−	+	+	+	+	−	−
A4 10	+	−	+	−	−	+	+	+	−	−	+	+	+	−	−
A4 11	+	+	+	−	−	+	+	+	−	−	+	+	+	−	+
A4 12	+	+	+	−	−	+	+	+	−	+	+	+	+	−	+
A4 13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Positivity rate (%)	100.0	75.0	62.5	50.0	20.8	100.0	87.5	100.0	45.8	54.2	100.0	91.7	100.0	70.8	50.0

Surprisingly, qPCR-positive pollen samples harboured significantly higher ($P < 0.001$) DWV loads (10^5–10^7 copies/µL) than honeybee samples (10^1–10^3, except one sample). Acute bee paralysis virus was detected in 12 (50%) samples by at least one of the primer pairs used (Tables 4 and 5). However, the ABPV-positive pollen samples were not consistent with the ABPV-positive bee samples, presumably because the ABPV-positive bees all had all come from one location (Apiary 4), while ABPV-positive pollen samples had been collected at all sampling locations (Table 3). Deformed wing virus was detected in 12 (50%) of the debris samples and BQCV and SBV in 17 (70.8%) of them, ABPV not being present in detectable quantities (Tables 2 and 3). While the viral loads of DWV detected in debris were lower than those in the pollen samples, ranging from 10^2 to 10^4 copies/µL, BQCV loads were comparable with those in all other types of materials. Surprisingly, SBV loads in the debris reached 10^9 copies/µL in three samples. As expected, detection of viruses in smear samples proved least successful because of the nature of the material used. Deformed wing virus was detected in just five (20.8%) of the smear samples, BQCV in 13 (54.2%), SBV in 12 (20%) and ABPV in 4 (16.6%) (Tables 2 and 3). As in bees, the frequency of positive DWV, BQCV and SBV samples was significantly higher in pollen and honey than in smears and debris (Table 2).

Table 3. Results of acute bee paralysis virus (ABPV) detection in bees, pollen, honey, hive debris and smears from the hive bottom board, using an end-point PCR

	ABPV				
Sample	Bees	Pollen	Honey	Debris	Smears
A1 1	–	–	–	–	–
A1 2	–	+	–	–	+
A1 3	–	+	–	–	–
A1 4	–	+	–	–	–
A2 1	–	–	–	–	–
A2 2	–	–	–	–	–
A2 3	–	–	–	–	–
A2 4	–	–	–	–	+
A2 5	–	+	–	–	–
A2 6	–	+	–	–	–
A3 1	–	+	–	–	–
A4 1	+	+	–	–	–
A4 2	+	+	–	–	–
A4 3	+	–	–	–	–
A4 4	+	+	+	–	–
A4 5	+	–	–	–	+
A4 6	+	–	+	–	–
A4 7	+	–	–	–	–
A4 8	+	+	–	–	–
A4 9	+	–	+	–	+
A4 10	+	+	–	–	–
A4 11	+	–	–	–	–
A4 12	+	+	–	–	–
A4 13	+	–	–	–	–
Positivity rate (%)	54.2	50.0	12.5	0.0	12.5

Table 4. Viral loads of deformed wing virus (DWV) in samples of bees, pollen, hive debris and hive grid smears

	DWV			
Sample	Bees	Pollen	Debris	Smears
A1 1	1.86×10^8	1.03×10^7	4.98×10^4	–
A1 2	5.26×10^2	7.60×10^5	2.90×10^4	3.50×10^5
A1 3	2.05×10^2	2.49×10^6	1.67×10^3	–
A1 4	1.13×10	–	2.54×10^3	1.01×10^5
A2 1	1.16×10^2	3.57×10^5	–	–
A2 2	9.64×10	–	–	–
A2 3	4.95×10	–	9.54×10^2	–
A2 4	7.58×10	–	7.15×10^2	–
A2 5	1.14×10^2	2.67×10^5	2.02×10^3	–
A2 6	8.49×10	–	–	–
A3 1	7.08×10	8.58×10^5	5.52×10^3	–
A4 1	–	1.42×10^6	–	–
A4 2	2.61×10	–	3.57×10^3	–
A4 3	1.94×10	–	–	–
A4 4	–	–	6.59×10^2	–
A4 5	3.83×10	–	1.80×10^2	–
A4 6	–	–	–	–
A4 7	–	1.11×10^6	–	–
A4 8	4.55×10	6.59×10^6	–	–
A4 9	7.41×10	–	–	–
A4 10	2.68×10^2	–	–	–
A4 11	2.72×10^2	–	–	–
A4 12	1.31×10^3	–	–	–
A4 13	6.96×10	–	–	–
Mean	$7.75 \times 10^{6\ a}$	$1.01 \times 10^{6\ ab}$	$4.03E \times 10^3$ ab	$1.89 \times 10^{4\ bc}$
SD	3.80×10^7	2.43×10^6	1.14×10^4	7.41×10^4

SD – standard deviation. Data represent numbers of virus copies/µL of analyte. Viral loads of some DWV-positive samples could not be calculated because of a lack of viral standards for the helicase-coding region targeted by one of the primer pairs used. – denotes a number of viral copies under the detection limit. Mean viral loads of materials with different lowercase letters are statistically significantly different ($P < 0.05$)

Table 5. Viral loads of BQCV and SBV in samples of bees, pollen, honey, hive debris and hive grid smears

	BQCV					SBV				
Sample	Bees	Pollen	Honey	Debris	Smears	Bees	Pollen	Honey	Debris	Smears
A1 1	4.23×10^7	1.58×10^7	2.83×10^6	7.21×10^5	–	1.76×10^4	5.86×10^6	1.01×10^6	8.39×10^6	–
A1 2	5.05×10^8	2.40×10^6	3.08×10^6	2.10×10^6	–	1.53×10^5	2.00×10^5	2.30×10^6	6.74×10^6	–
A1 3	5.24×10^7	3.28×10^6	3.22×10^6	1.43×10^5	–	1.64×10^5	2.06×10^6	3.81×10^6	3.98×10^6	–
A1 4	5.57×10^7	2.85×10^5	2.15×10^6	7.58×10^6	1.36×10^5	3.10×10^4	4.53×10^4	1.70×10^6	1.04×10^7	–
A2 1	5.66×10^6	–	1.03×10^6	–	7.87×10^5	3.95×10^4	–	4.96×10^5	4.26×10^8	2.27×10^4
A2 2	3.14×10^6	5.00×10^6	2.03×10^5	–	–	1.41×10^9	1.22×10^6	7.19×10^4	3.31×10^7	–
A2 3	7.56×10^6	5.42×10^6	3.78×10^5	5.94×10^6	–	9.00×10^8	4.41×10^6	2.09×10^5	1.08×10^9	1.19×10^8
A2 4	5.66×10^7	7.62×10^5	2.30×10^6	–	4.18×10^5	2.30×10^8	3.00×10^5	1.99×10^6	1.20×10^9	2.46×10^5
A2 5	2.10×10^7	8.70×10^6	2.23×10^6	1.27×10^6	7.98×10^5	8.48×10^9	1.57×10^7	1.79×10^6	2.94×10^9	4.41×10^6
A2 6	9.89×10^7	–	3.59×10^6	7.24×10^5	7.99×10^5	3.06×10^6	7.91×10^4	1.48×10^6	8.68×10^6	3.53×10^5
A3 1	1.40×10^7	2.81×10^6	4.17×10^6	7.70×10^6	–	4.73×10^5	1.08×10^6	2.33×10^6	5.06×10^7	2.14×10^4
A4 1	3.94×10^7	3.71×10^7	4.94×10^6	–	–	2.96×10^5	8.30×10^6	8.07×10^5	–	–
A4 2	1.90×10^8	2.37×10^7	1.75×10^6	2.63×10^6	2.28×10^5	4.20×10^8	4.20×10^6	1.71×10^5	8.60×10^7	–
A4 3	6.86×10^7	2.62×10^7	1.69×10^5	–	–	1.01×10^5	4.21×10^6	4.26×10^4	1.40×10^7	–
A4 4	4.74×10^7	5.59×10^6	3.40×10^5	–	–	2.09×10^5	1.33×10^6	6.47×10^4	1.64×10^7	–
A4 5	1.16×10^8	1.52×10^7	3.57×10^6	1.05×10^6	–	1.22×10^5	3.29×10^6	6.65×10^5	1.08×10^6	8.41×10^3
A4 6	4.93×10^7	–	4.86×10^6	–	6.87×10^4	2.91×10^5	–	1.69×10^6	5.34×10^6	–
A4 7	1.07×10^8	3.50×10^7	1.48×10^6	–	–	1.16×10^5	4.60×10^6	2.38×10^5	–	–
A4 8	1.48×10^8	8.35×10^7	1.10×10^6	–	5.03×10^5	1.12×10^5	4.61×10^6	1.07×10^5	–	1.05×10^5
A4 9	1.62×10^8	2.45×10^7	7.64×10^4	–	2.42×10^5	6.43×10^8	1.95×10^6	1.58×10^4	–	–
A4 10	9.39×10^7	2.40×10^7	1.90×10^6	–	–	3.51×10^5	2.93×10^6	7.64×10^5	–	–
A4 11	7.00×10^7	2.25×10^7	1.16×10^7	–	–	4.20×10^5	2.19×10^7	4.43×10^6	–	9.20×10^3
A4 12	2.82×10^8	3.74×10^7	3.55×10^5	–	1.60×10^5	4.10×10^5	1.10×10^7	5.64×10^4	–	4.56×10^4
A4 13	1.00×10^8	3.32×10^7	1.15×10^6	5.84×10^5	2.59×10^5	1.72×10^9	8.10×10^6	2.88×10^5	4.19×10^8	7.95×10^5
Mean	$9.73 \times 10^{7\ a}$	$1.72 \times 10^{7\ b}$	$2.44 \times 10^{6\ bc}$	$1.27 \times 10^{6\ cd}$	$1.83 \times 10^{5\ d}$	$5.75 \times 10^{8\ a}$	$4.47 \times 10^{6\ a}$	$1.11 \times 10^{6\ a}$	$2.63 \times 10^{8\ a}$	$5.21 \times 10^{6\ b}$
SD	1.09×10^8	1.93×10^7	2.46×10^6	2.37×10^6	2.75×10^5	1.75×10^9	5.38×10^6	1.22×10^6	6.58×10^8	2.43×10^7

SD – standard deviation. Data represent numbers of virus copies/μL of analyte. – denotes a number of viral copies in samples under the detection limit. Mean viral loads of materials with different lowercase letters are statistically significantly different ($P < 0.05$)

Discussion

Honey bee viruses were detected in all four types of non-invasive material tested. None of these materials are routinely used for detection of honey bee viruses, the majority of previous research on transmission of viruses *via* honey bee products having been focused on pollen and its potential for inter-taxa infection (9, 16, 27). While all the major honey bee viruses have previously been detected in pollen particles (2, 3, 4, 16, 19, 27, 28), some have also been detected in other bee products (25, 29). For example, honey, pollen and wax have all been reported as sources of viral infection, with no significant difference in infectivity between the materials (23).

In addition to facilitating non-invasive screening, another advantage of some of the materials studied in the present work is easier processing. While the hard cuticles of the bee samples, for example, needed to be crushed during the homogenisation process (7), there was no need for homogenisation of the honey and pollen samples due to their natural consistency. On the other hand, each material carried some disadvantages, with the smear samples being the least reliable sample type. Swabs are routinely used in veterinary medicine to obtain virus-containing samples from different areas according to the clinical symptoms (*e.g.* from skin lesions and nasal, conjunctival and urogenital regions) (10). In such cases, however, the swabs are taken directly from a diseased animal, usually in the form of a bodily fluid sample containing viral particles. The approach used in this study was different in that the swabs were used to take samples from the environment (*i.e.* the metal grid in the hive bottom board where

debris and dead honey bees fall) rather than from the animal itself, and as such, the collection method was not as reliable as that used in a veterinary setting. While we were able to detect some virus-positive samples, the method would need to be refined and thoroughly tested in order to be usable, *e.g.* by swabbing more areas of the hive and comparing these to determine the practicability of the sample material. Multiple smears were not taken from different hive parts in this study as the main aim was to assess whether it was possible to detect honey bee viruses in smears, the method not having been used before to our knowledge.

Detection and quantification of viruses in debris was much less successful than detection in honey or pollen. Hive debris consists mostly of wax from uncapped honey cells or wax particles that fall when the newly emerging bees chew through the cell capping. However, it also commonly contains varying proportions of other materials, such as pollen loads lost by the bees, sugar crystals or small parts of dead bees that fall through the grid in the bottom board. The heterogeneity of such materials could lead to inconsistent results during virus detection. However, the main drawback of virus detection from debris is the laboriousness of the nucleic extraction process, particularly as regards the wax particles, which melt easily, making it very difficult to work with. Consequently, the extracted nucleic acids need to undergo a secondary refining step in order for qPCR to run correctly; detection being far less successful without this step (20).

Of all the non-invasive materials tested, the honey and pollen samples provided best results, with the viral loads for BQCV and SBV being particularly high and almost reaching those detected in the control (bee) samples. In comparison, the detection levels of the other viruses were lower, DWV, for example, being omnipresent in the bee samples but not detected in any honey or pollen samples. This could have been due to viral titres being below the detection threshold, as some of the bee samples contained very low concentrations of viral particles per µL. The results for ABPV detection were the least consistent, bee samples testing positive for ABPV at just one location, Apiary 4, and the few honey samples where ABPV was detected also originating from the same apiary. In the case of pollen samples, however, the results were completely different, with at least one pollen sample from Apiaries 1, 2 and 3 found to be ABPV-positive, despite bees from these localities being ABPV-negative. There are several possible explanations for this inconsistency. Both DWV and ABPV can infect not only honey bees but other insect pollinators as well (5, 9, 11). As the bees share the local pastures with other wild pollinators and honey bee colonies, they are likely to come into direct contact with pollen and flowering plant structures visited by infected pollinators, which act as a transmission route for the bee-infecting viruses (1). Unlike nectar, however, pollen does not undergo any enzymatic processes inside the honey sac and does not come into direct contact with the forager's digestive system before storage; thus, potentially contaminated pollen can be brought inside the hive and stored without infecting the bee. This may explain the absence of a detectable ABPV load in the pooled bee samples and its presence in beehive pollen samples. Moreover, the foraging bees collected in this study only consume very low amounts of the pollen compared to the larvae and nurse bees (6). Another partial explanation may be the nutritional quality and antiviral activity of phytochemicals found in the pollen (17). While high-quality nutrition is closely linked with honey bee wellness and immunity, there is no correlation between pollen quality and the presence of pathogens (including viruses), *i.e.* the presence of a virus does not affect the nutritional value of the pollen (28). Thus, the pollen potentially provides quality nutrition that allows the bee to thrive despite the presence of the virus. To summarise, pollen does not come into direct contact with the digestive structures of the forager bee, foragers only consume low amounts of the pollen collected, pollen consumption does not usually lead to the development of overt infection and/or high viral loads, and the nutritional and antiviral properties of pollen act as a "regulator" of the development of viral diseases (17).

Acute bee paralysis virus is strongly associated with the parasitic *Varroa* mite, which acts as a vector for many viruses; indeed, high viral loads are mainly found in bees infected *via* this vector-mediated route (8). The samples used in this study were collected in late spring, when the extensively produced brood is abundant and the majority of mites are present on the capped brood (22). Consequently, the majority of vector-infected individuals would still have been in their developmental stages at collection time. Owing to the high virulence of the virus, infected broods often succumb to the infection before emergence, while infected adult bees die within a few days (24); thus, most infected bees do not live long enough to become foragers, *i.e.* the bees collected for the pooled samples in the present work. The high virulence of ABPV is also noted as one possible reason why it is less frequently detected in bees than DMV (24).

It is also possible that the bees in Apiary 4 were influenced by some factor that provided improved conditions for ABPV multiplication, or that the ABPV strain present at this locality was more successful in replication and/or matched our primers better. However, all the bee samples used came from apparently healthy and strong colonies, indicating that the detectable (*i.e.* higher) ABPV loads had not had any detrimental effect on colony performance.

In conclusion, honey bee viruses were successfully detected in a range of non-invasive materials sourced from the hive (*i.e.* honey, pollen, debris and smears) and the levels compared with those

detected in control samples of forager honey bees from the same hive. Overall, honey and pollen samples provided the best comparative results; however, the use of these materials needs to be refined and more testing is needed to ensure reliable results. It would also be useful to include additional control groups comprising nurse bees and brood in future testing as their viral profile will differ from that of the foragers (18).

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