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Validation of quantitative real-time RT-PCR assays for the detection of six honeybee viruses

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ABSTRACT

Acute bee paralysis virus (ABPV), *Black queen cell virus* (BQCV), Chronic bee paralysis virus (CBPV), *Deformed wing virus* (DWV), *Sacbrood virus* (SBV) and *Varroa destructor virus 1* (VDV1) are the six main honeybee viruses reported in Europe. We assessed the accuracy (trueness and precision) of reverse transcriptase quantitative TaqMan® PCR methods (RT-qPCR) for quantifying ABPV, BQCV, DWV, VDV1 and SBV loads. Once the systematic bias in quantitative results had been corrected (overestimation in ABPV and BQCV quantification and underestimation in that of SBV and VDV1), measurements were taken to determine the viral load ranges for which quantification uncertainty was below ± 1 $\log_{10}$ equivalent of genome copies per bee (hereafter reported as genome copies/bee). The accuracy range of RT-qPCR was found to be between 6.4 and 10.4 $\log_{10}$ genome copies/bee for ABPV, between 3.0 and 10.0 $\log_{10}$ genome copies/bee for BQCV, between 2.4 and 10.4 $\log_{10}$ genome copies/bee for DWV and between 3.4 and 10.4 $\log_{10}$ genome copies/bee for SBV. Outside these ranges, the results' uncertainty is higher. VDV1 RT-qPCR accuracy was outside accuracy limits for all viral loads. Using these RT-qPCR methods, we quantified viral loads in naturally-infected honeybees. The viral load distribution and clinical signs reported with the honeybee samples allowed us to define a threshold that could be used to differentiate between covert and overt infections. These methods will be useful in diagnosing the main viral infections impairing honeybee health.

1. Introduction

As many as 15 distinct honeybee virus species-complexes have been reported to infect honeybees in nature with one or more strains or subspecies (de Miranda et al., 2015). The most recently-discovered viruses were found through new-generation sequencing and their implications for honeybee health need to be further investigated (McMenamin and Flenniken, 2018). Among the viruses that mainly affect honeybee heath, six are associated with colony disorders in European honeybees. *Acute bee paralysis virus* (ABPV; family: *Dicistroviridae*) normally persists at low viral loads with no obvious symptoms (covert infection). However, under certain conditions ABPV can become extremely virulent, reducing adult honeybee and brood populations without any clinical signs being observed in the field (de Miranda and Genersch, 2010). When honeybees were experimentally inoculated with ABPV, paralysis and death were observed five days later (Bailey et al., 1963). Losses can be greater if colonies are infested by *Varroa destructor*; this honeybee mite is a vector of ABPV, although there is no evidence of viral replication in the mite (de Miranda et al., 2010). Larvae infected by *Sacbrood virus* (SBV; family: *Iflaviridae*) stop pupating and fluid accumulates between the larva's body and unshed skin, forming a saccule. Dead larvae can also be indicated by a scattered brood. SBV can decrease the lifespan of infected foragers (Bailey and Fernando, 1972). *Black queen cell virus* (BQCV; family: *Dicistroviridae*) seems to be less harmful than SBV. It kills developing queen larvae and turns the cells black. However, its pathology in adult and brood populations seems closely related to co-infections with the microsporidia *Nosema apis* and *Nosema ceranae* (Bailey et al., 1983; Doublet et al., 2015).

For ABPV, BQCV and SBV, it has been suggested that adult death occurs during foraging and that dead larvae are cannibalised by other honeybees, thereby explaining an absence of honeybee or larva corpses in front of weak colonies (de Miranda et al., 2010). Chronic bee paralysis virus (CBPV; unclassified) induces trembling and cuticle melanisation (black bees) before causing the death of numerous foragers over a short period of time, such that large numbers of dead adult bees are found at the hive entrance (Ribiere et al., 2010). *Deformed wing virus*

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Table 1
Primers, probe and recombinant plasmids used for RT-qPCR quantification.

Virus	Forward primer (5' - > 3')	Reverse primer (5' - > 3')	Probe (5' - > 3')	Reference	Primer concentrations	Probe concentration
ABPV	CATATTGGCGAGCCACTATG	CTACCAGGTTCAAAGAAAATTTC	(6-Fam) ATAGTTAAAACAGCTTTTCACACTGG (Tamra)	(Jamnikar-Ciglenecki and Toplak, 2012)	800 nM	100 nM
BQCV	GGTGCGGGAGATGATATGGA	GCCGTCTGAGATGCATGAATAC	(6-Fam) TTTCCATCTTTATCGGTACGCCGCC (Tamra)	(Chantawannakul et al., 2006)	320 nM	200 nM
CBPV	CGCAAGTACGCCTTGATAAAGAAC	ACTACTAGAAACTCGTCGCTTCG	(6-Fam) TCAAGAACGAGACCACCGCCAAGTTC (Tamra)	(Blanchard et al., 2012)	320 nM	200 nM
DWV	GCGGCTAAGATTGTAAATTG	GTGACTAGCATAACCATGATTA	(6-Fam) CCTTGACCAGTAGACACAGCATC (Tamra)	This study	350 nM	100 nM
SBV	AAGGTCCACTACACCGAAATGTC	ACACTGCGGTCTAACATTCC	(6-Fam) TGATGAGAGTGGACGAAGA (MGB)	(Blanchard et al., 2014)	320 nM	200 nM
VDV1	GGTCTGAAGCGAAAATAG	CTAGCATATCCATGATTATAAAC	(6-Fam) CCTTGTCCA GTAGATACAGCATCACA (Tamra)	This study	1,200 nM	400 nM

Plasmid name	Plasmid vector (supplier)	Cloned sequence: nucleotide position	Virus (GenBank accession number)	Primers (Forward/Reverse)	Reference
pB2	pCR II Topo (Invitrogen)	8115 to 8512	ABPV (AF126050)	ABPV1/ABPV2	(Bakonyi et al., 2002)
pNC1-4	pGEM-T Easy (Promega)	7850 to 8550	BQCV (AF183905)	BQCV1/BQCV2	(Benjeddou et al., 2001)
pAb2	pGEM-T Easy (Promega)	2260 to 3059	CBPV (EU122229)	R3_RC/CBPV3'	(Blanchard et al., 2007)
pC1	pCR II Topo (Invitrogen)	4240-4659	DWV (AY292384)	DWV3/DWV4	(Blanchard et al., 2007)
pD1	pCR II Topo (Invitrogen)	241-669	SBV (AF092924)	SBV1f/SBV2r	(Grabensteiner et al., 2001)
pFab1	pUC57 (Gen Cust)	2551-4573	VDV1 (AY254569.2)	Gene synthesis (Gen Cust)	This study

(DWV) and *Varroa destructor virus 1* (VDV1) belong to the same viral cloud (DWV type A and DWV type B, respectively (Martin et al., 2012); family: *Iflaviridae*). Both viruses cause wing deformities in developing honeybees that die shortly, and reduce the lifespan of infected adult bees (Benaets et al., 2017; Brettell et al., 2017; de Miranda and Genersch, 2010). Clinical signs are worsened by *V. destructor* infestation. Indeed, DWV and/or VDV1 combined with an infestation of this mite are predictive markers of European colony losses (Dainat and Neumann, 2013).

Since all these viruses are RNA viruses, they can be detected by conventional or real-time RT-PCR, and several methods have been described in the literature (the Beebook provides a review of methods that can be used (de Miranda et al., 2013). Because honeybee viruses can infect colonies without inducing any visible clinical signs (covert infection), and because multiple viral infections are often reported (Amiri et al., 2015; Gauthier et al., 2007), the aetiological cause of the disorders observed could be indicated by estimating honeybee viral loads through quantitative RT-PCR (RT-qPCR) (Amiri et al., 2015). An increase in viral load indicates a breakdown in the virus/host balance, leading to perceptible clinical signs (overt infections) and sometimes to honeybee death, followed in the worst cases by colony loss. Few of the reported RT-qPCRs have been fully validated to be use as official methods according to the European Union's regulation 2017/625 (European Union, 2017). Until now, CBPV-RT-qPCR has been the most fully validated method (Blanchard et al., 2012). Its accuracy (trueness and precision) was established according to French standard NF U47-600 (AFNOR, 2015) and the reliability of viral load calculation was evaluated through inter-laboratory proficiency testing (Schurr et al., 2017). A diagnostic threshold was set at $\geq 10^8$ copies/bee to establish a link between the honeybee disorders observed and CBPV infection (Blanchard et al., 2007). SBV, ABPV and BQCV RT-qPCRs have also been characterised, but the accuracy of their viral loads had not been estimated previous to this study. For DWV and VDV1, which produce identical clinical signs, few RT-qPCRs have been able to distinguish between the two viruses (Bradford et al., 2017; Kevill et al., 2017).

The French National Reference Laboratory, which is also the European Union Reference Laboratory for Honeybee Health, is responsible for validating diagnosis methods for use by official laboratories. In this study, we describe the accuracy (trueness and precision) of five quantitative TaqMan® RT-qPCRs in quantifying ABPV, BQCV, DWV, SBV and VDV1 in honeybees. We furthermore present and analyse the viral loads quantified in honeybees using these methods and the CBPV RT-qPCR method which had previously been validated (Blanchard et al., 2012) in order to recommend thresholds to be used to distinguish between covert and overt infections.

2. Material and methods

2.1. Naturally-infected honeybees

Our laboratory received samples for viral diagnosis (569 adult honeybee samples and 81 larvae or pupae samples). More than half of the samples (373 samples) had been collected (between 2012 and 2019) from French apiaries where clinical signs had been reported by veterinarians or beekeepers. Another 123 samples had been collected from apiaries without clinical signs, and a further 154 samples were received without clear information about the colonies' health. In order to describe the viral status of the colonies, viral loads were determined (n = 1,735) using pooled samples from the same colony. Ten bees (or ten larvae) per colony were crushed in 10 ml of 10 mM phosphate buffer (pH 7.0), which was clarified by two successive centrifugations lasting 10 min at 8000 x *g* as previously reported (Blanchard et al., 2007). Supernatants were stored at −80 °C before testing.

2.2. Primers, probes and recombinant DNA plasmids

The primers and probes used to quantify ABPV, BQCV, CBPV and SBV by RT-qPCRs had previously been published (Table 1). For DWV and VDV1, we designed new primers and probes able to distinguish one virus from the other in coding sequence VP3. VDV1's VP3 sequence was also found in the genomes of recombinant viruses between DWV and VDV1 (Dalmon et al., 2017). Primer and probe concentrations were adjusted in order to optimise PCR efficiency (between 80 and 120%; (AFNOR, 2015)).

Six recombinant plasmids (Table 1) were produced in order to establish the standard curves of RT-qPCRs and to be used as a template for validation tests. The plasmids were quantified by UV spectrometry and 10-fold serial dilutions were prepared in 100 mM Tris, 50 mM EDTA, pH 8.0 buffer (TE buffer).

2.3. Harmonised method for quantifying honeybee viruses

The methods were harmonised for viral RNA purification and TaqMan® two-step RT-qPCR. For ABPV, BQCV, DWV and SBV quantification, conditions were based on the method used to quantify CBPV using RT-qPCR (Schurr et al., 2017). Briefly, total RNAs were purified from 200 µl of clarified bee homogenate using the High Pure Viral RNA Kit (Roche Diagnosis) according to the manufacturer's instructions. The RNAs were recovered from a spin column in 50 µl of elution buffer. Complementary DNA (cDNA) was synthesised at 42 °C for 1 h in reverse transcriptase buffer (50 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.5 mM dNTP, 20 pmol of random hexamer primers, 20 U of RNase Out (Invitrogen), 200 U of SuperScript II reverse transcriptase (Invitrogene) and 12.5 µl of extracted RNA, in a total volume of 20 µl. The amplification reaction was subsequently performed in a MicroAmp optical 96-well reaction plate containing 1X TaqMan® Universal PCR Master Mix with uracil-*N*-glycosylase (UNG) (2X, Applied Biosystems), 320 nM to 1200 nM of each forward and reverse primer, 100–400 nM of the probe, 1X Exo IPC Mic VIC (10X, Applied Biosystems), 1X Exo IPC DNA (50X, Applied Biosystems) and 5 µl of cDNA template in a final volume of 25 µl. For VDV1 qPCR the amplifications were performed without internal positive control (without 1X Exo IPC Mic VIC or 1X Exo IPC DNA). For CBPV quantification, two samples of cDNA (5 µl) were amplified to calculate a Ct mean (Schurr et al., 2017).

The thermal cycling conditions were also harmonised with CBPV qPCR: 2 min at 50 °C (active temperature for UNG to degrade any carryover DNA amplified from previous reactions), 10 min at 95 °C (activation of AmpliTaq Gold DNA Polymerase and degradation of UNG), followed by 40 denaturation cycles at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

Threshold cycles (Ct) from our samples were compared with standard curves establishing a linear relation between Ct and plasmid loads in the range of 1.0×10^2 to 1.0×10^9 copies/5 µl (loads in $\log_{10}$). Results were expressed in equivalent viral genome copies per bee (genome copies/bee) using the following equation: Copies/bee = 80 × copies/PCR. This conversion factor was calculated based on the volume used at each step of the RT-qPCR method (Schurr et al., 2017).

2.4. Spiked honeybee samples

Emerging bees were collected from colonies in our winter apiary. They all tested negative for ABPV, BQCV and SBV by RT-qPCR. For DWV and VDV1 experiments, honeybees from the Åland Islands (an area free of *V. destructor*) were provided by Doctor Eva Forsgren (Swedish National Reference Laboratory for honeybee health). Honeybees were crushed in phosphate buffer (1 ml per bee) and the homogenate was clarified as previously described. The bee homogenates were spiked with known quantities of recombinant plasmids (targeted by qPCRs; Table 1). Samples were processed according to viral load quantification methods.

2.5. Efficiency and linearity of quantitative PCRs

For each qPCR-trial, six standard curves were generated by linear regression analysis of the Ct vs. the $\log_{10}$ copy number for recombinant plasmids (between 2.0–8.0 $\log_{10}$ copies/PCR). The linear regression line is given by the formula $Ct = a[\log_{10}(x)] + b$, where a is the slope, b is the y-intercept, and x is the standard quantity. Amplification efficiency (E) was calculated with slope (a) of the linear regression for each standard curve, using the formula $E = 10^{|1/a|} - 1$.

To assess linearity, measured plasmid loads were retrospectively determined using the formula: $\log_{10}(x) = [Ct - b]/a$. The values thus obtained were compared to the theoretical quantities of plasmid and provided the mean bias. In accordance with French standard NF U47-600 (AFNOR, 2015), the maximum allowed bias for the measured quantities was set at ± 0.25 $\log_{10}$/PCR.

2.6. Limit of detection, limit of quantification and accuracy profile for quantification methods

The limit of detection (LOD) for the RT-qPCR method was the amount of biological target initially present in the sample to be detected. The LOD was determined as being the last dilution in which recombinant plasmid can be detected in all tested replicates (n = 8).

The limit of quantification (LOQ) and the methods' accuracy were assessed through the construction and interpretation of an accuracy profile designed to estimate the precision and reliability of the values (Feinberg, 2007). For each dilution series and each viral load, the quantities actually obtained were compared to the theoretical quantity. Various parameters were defined in order to construct the accuracy profile, including the standard deviation of repeatability (SD_r), standard deviation of reliability (SD_R) and mean bias between the theoretical value and mean of the values obtained (NF U47-600; AFNOR, 2015). To analyse the accuracy profile, the quantification methods' lower and upper tolerance limits (95% confidence interval) were determined using the following formula: upper limit = mean bias + 2 × SD_R; lower limit = mean bias-2 × SD_R. They were then compared to the accuracy limits defined as ± 1.0 $\log_{10}$ plasmid copies/bee. The LOQ is the last dilution in which the plasmid load can be quantified with acceptable accuracy.

3. Results

3.1. Quantitative PCR performance

The quantification bias of five viral qPCRs was assessed using 10-fold serial dilutions of recombinant plasmid diluted in TE buffer (Fig. 1). The plasmid copy numbers quantified by all the qPCRs were within the range of expected loads ± 0.25 $\log_{10}$ for each plasmid level between 2.0 and 8.0 $\log_{10}$ copies/PCR. The PCR efficiencies were calculated to be 86% for ABPV-qPCR, 105% for BQCV-qPCR, 104% for DWV-qPCR, 92% for SBV-qPCR and 84% for VDV1-qPCR.

3.2. RT-qPCR method performance

The LODs of the viral RT-qPCR methods were estimated using honeybee homogenates spiked with serial dilutions of plasmid (eight replicates per load). The LODs were estimated to be 4.4 for ABPV qPCR, 3.0 for BQCV qPCR, 2.4 for DWV qPCR, 3.4 for SBV qPCR and 5.0 for VDV1 qPCR (values expressed in $\log_{10}$ plasmid copies/bee).

The quantity of plasmids, measured by the RT-qPCRs in honeybee homogenates spiked with 10-fold serial dilutions of plasmid (six replicates per load), were used to estimate the accuracy of the viral quantitative methods (Fig. 2). The DWV method estimated plasmid load with trueness (bias = 0 $\log_{10}$ copies/bee) and precision ($2xSD_R$ = 0.27 $\log_{10}$ copies/bee). The LOQ was estimated to be 2.4 $\log_{10}$ plasmid copies/bee. For ABPV, BQCV, SBV and VDV1, systematic quantification

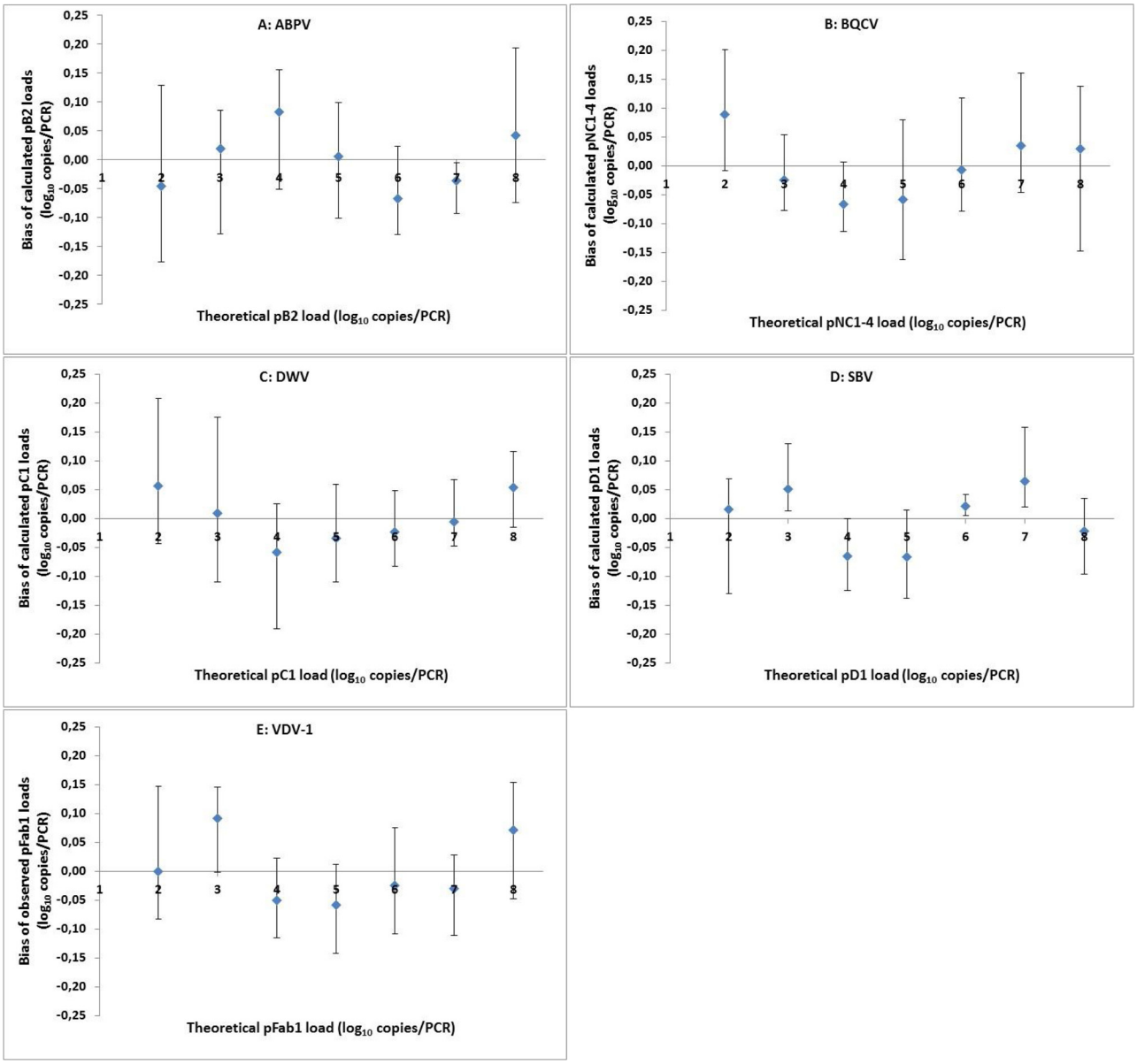

Fig. 1. Quantification bias of ABPV, BQCV, DWV, SBV and VDV1 qPCRs. Six standard curves were generated for each viral qPCR (A to E) and used to calculate the mean bias between the quantity found by qPCR and the theoretical plasmid load (copies per PCR). The bars show the maximal and minimal bias calculated for each plasmid load.

bias was found. The plasmid loads found in honeybees were underestimated by both the VDV1 method (mean bias = -0.95 $\log_{10}$ copies/bee) and the SBV method (mean bias = -0.4 $\log_{10}$ copies/bee). The ABPV and BQCV methods systematically overestimated plasmid loads in honeybee homogenates (ABPV: mean bias = $+0.4$ $\log_{10}$ copies/bee; BQCV: mean bias = $+0.2$ $\log_{10}$ copies/bee). Consequently, the estimated plasmid load accuracy was outside limits ($\pm$ 1.0 $\log_{10}$ plasmid copies/bee). When these systematic biases (lack of trueness) were corrected, the accuracy of the ABPV, BQCV and SBV quantitative methods fell within accuracy limits (Fig. 2). The mean reproducibility of the corrected plasmid loads (in the validated plasmid load range) was calculated to be 0.63 for ABPV, 0.30 for BQCV and 0.41 for SBV (expressed in $\log_{10}$ plasmid copies/bee). The LOQs were estimated to be 6.4, 3.0 and 3.4 $\log_{10}$ plasmid copies/bee respectively. Despite such a correction of the systematic bias, the VDV1 method's accuracy was outside accuracy limits. The corrected plasmid loads' mean reproducibility was determined to be 0.87 $\log_{10}$ copies/bee for the plasmid load range of 5–10 $\log_{10}$ copies/bee.

3.3. Viral loads quantified in naturally-infected honeybee samples and viral load thresholds

The frequencies of viral loads determined in naturally-infected honeybees (or larvae) by the five RT-qPCR methods are shown in Fig. 3. For ABPV, CBPV, DWV, VDV1 and SBV the viral distributions showed two groups. For each bimodal distribution, a threshold was set at the lower frequency between both groups. For ABPV loads determined in honeybees (n = 84), most of the samples were pooled adult bees. Two groups of ABPV loads were observed: the first group below (n = 49; 58%) and the second above (n = 35; 42%) a viral load threshold set at 5 $\log_{10}$ genome copies/bee or larva. The ABPV loads quantified in the three brood samples were below 4 $\log_{10}$ genome copies/larva. CBPV loads were measured only in adult honeybees (n = 296). The first group of honeybees consists of those infected with CBPV at a level below the threshold set at 8 $\log_{10}$ genome copies/bee (n = 143; 48%). The second group is made up of honeybees with viral loads above this threshold (n = 153; 52%). DWV loads and VDV1 loads were measured in samples

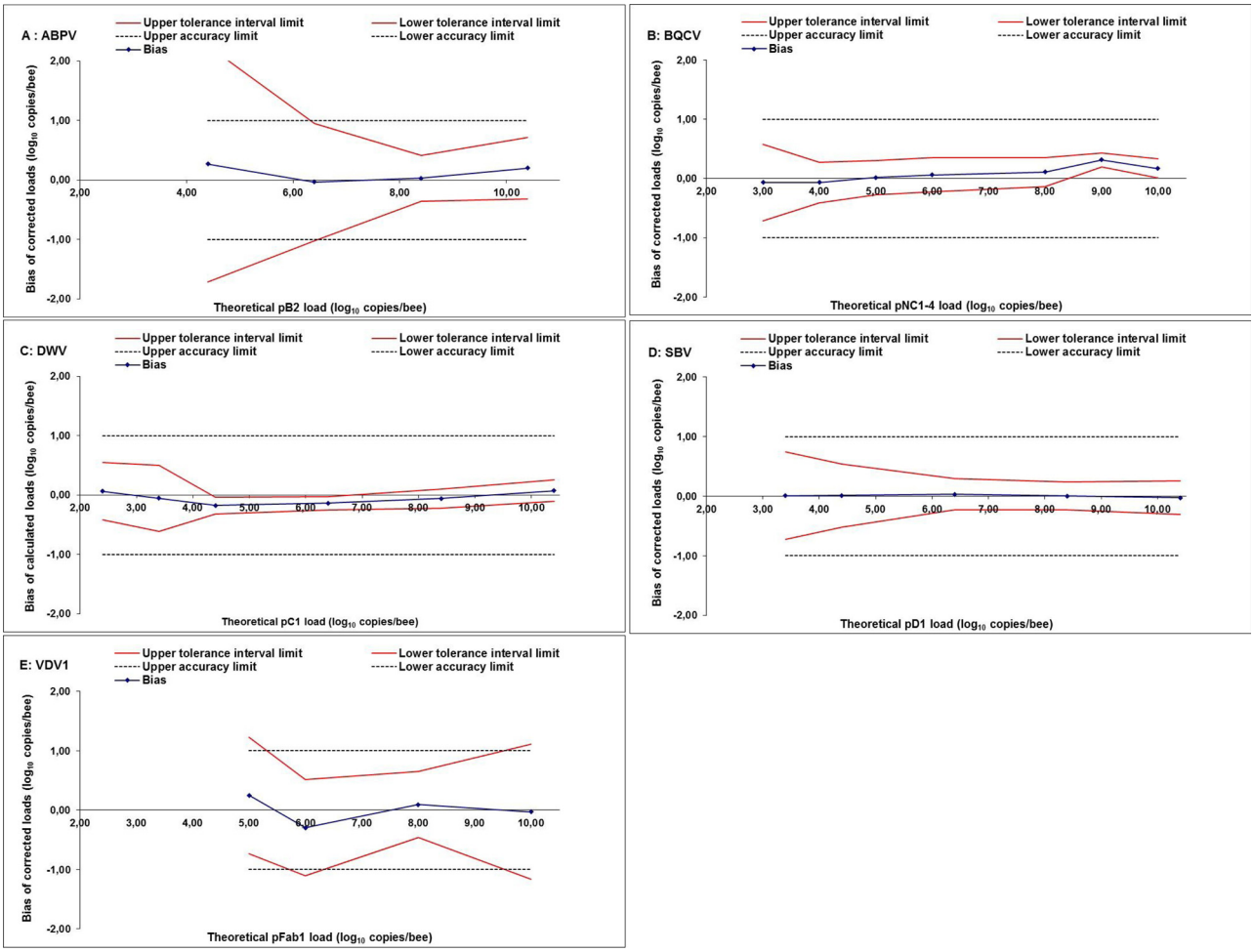

Fig. 2. Accuracy profiles of ABPV, BQCV, DWV, SBV and VDV1 quantification methods. Plasmids in honeybee homogenates were quantified by the RT-qPCRs (A to E). Accuracy limits were set at ± 1.0 $\log_{10}$ plasmid copies/bee. Upper and lower tolerance interval limits were determined for each load from the mean bias ± twice the standard deviation of calculated plasmid loads. The quantified ABPV, BQCV, SBV and VDV1 plasmid loads were corrected for systematic bias (+0.4, +0.2, −0.95 and −0.4 respectively).

of brood (n = 18 and n = 1, respectively) and adult honeybees (n = 121 and n = 87, respectively). Two groups were found: below and above a viral load thresholds set at 6 $\log_{10}$ DWV genome copies/bee or larva and at 7 $\log_{10}$ VDV1 genome copies/bee or larva. Each group contained both brood and adult honeybee samples. Since 2014 and out of 75 DWV loads measured by RT-qPCR, only one sample (1%) has been quantified with a DWV load above the threshold (7.63 $\log_{10}$ genome copies/bee). Both groups of DWV-variant loads have been quantified since 2017 across 88 samples. VDV1 loads above 7 $\log_{10}$ copies/bee or larva were found in 53 of them (60%). For SBV-load determination, two groups were again found: the first group below (n = 168; 85%) and the second above (n = 29; 15%) a viral load threshold falling between both groups, at 9 $\log_{10}$ genome copies/bee or larva. Brood (n = 37) and adult honeybee (n = 160) samples were found in both groups.

When BQCV loads in brood (n = 34) and adult honeybee (n = 192) samples were quantified, just one group was found. The BQCV loads followed a normal distribution; the distribution limit which included 97.5% of the results was calculated to be 8 $\log_{10}$ BQCV genome copies/bee or larva (two standard deviations of the mean). Nine brood samples (26%) and four adult samples (2%) displayed a load above this threshold.

3.4. Clinical signs reported for honeybee samples with viral loads above the thresholds

The samples received with colony health data reported by veterinarians or beekeepers were used to determine the clinical signs most frequently associated with samples presenting viral loads above the viral thresholds. For samples whose ABPV loads were above 5 $\log_{10}$ genome copies/bee (n = 27), many beekeepers or veterinarians had reported colony health issues (78%). However, samples with high ABPV loads had also been collected from healthy colonies (6%); notably one bee sample was quantified at 9.05 $\log_{10}$ genome copies/bee. The most frequently observed clinical sign was colony depopulation (38%). One sample with a viral load of 10.03 $\log_{10}$ genome copies/bee had been collected from a depopulated colony with trembling bees (moreover, the CBPV genome was also quantified in this sample at 6.47 $\log_{10}$ copies/bee). For BQCV, 92% of the samples quantified with a viral load above 8 $\log_{10}$ genome copies/bee or larva had been collected from colonies with health issues. Four samples displayed unusually-coloured capped cells. For CBPV loads quantified above 8 $\log_{10}$ genome copies/bee, 100% of honeybee samples were collected from colonies with health disorders (n = 134). Mass honeybee death at the hive entrance was frequently observed (90%), as well as trembling or crawling bees (87%) and black bees (47%). Of the samples collected from colonies showing no clinical signs (n = 105), none were quantified with a viral load above 6 $\log_{10}$ genome copies/bee.

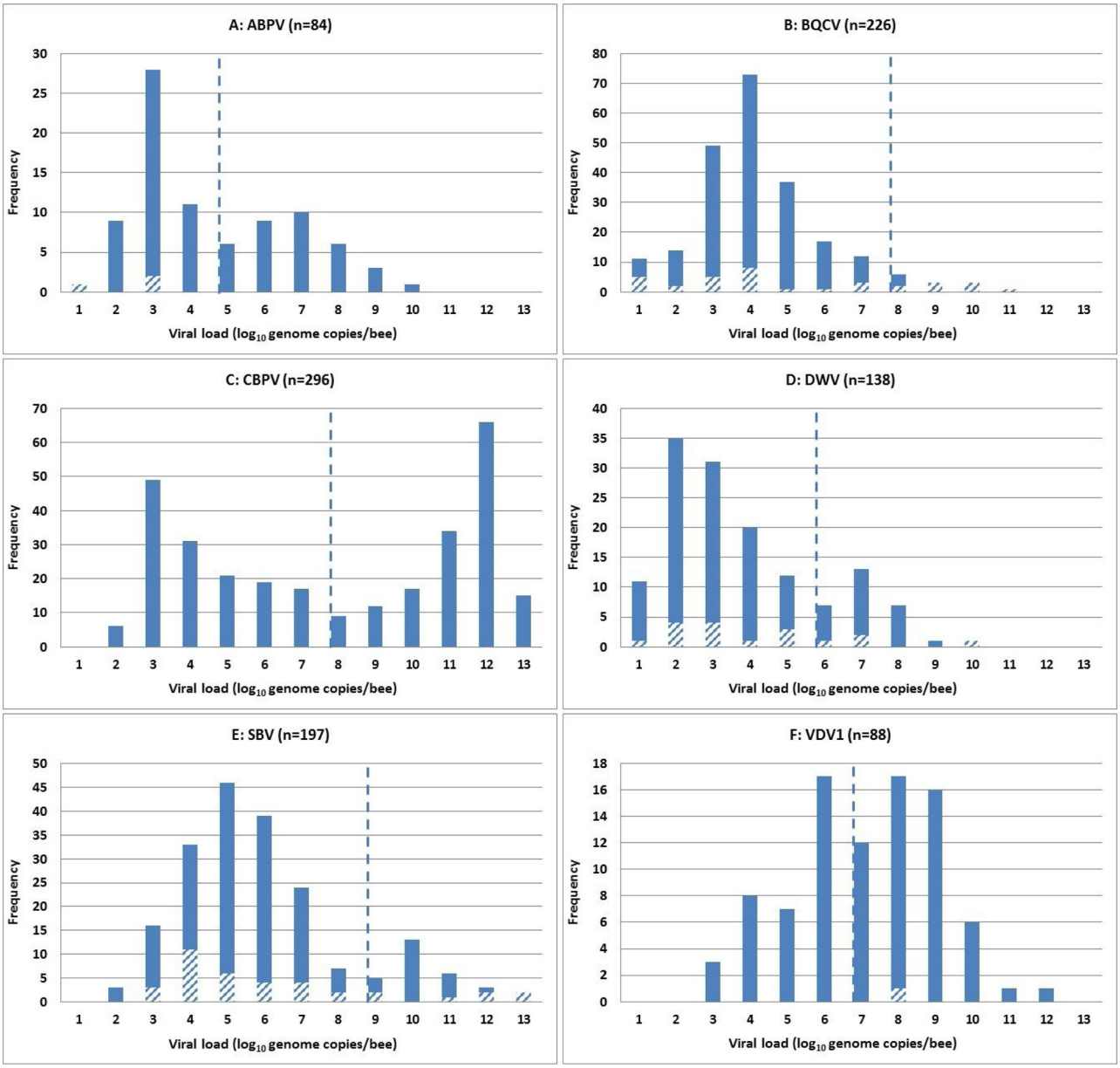

Fig. 3. Frequency of viral load detection in samples of naturally-infected honeybees. Viral loads quantified in adult bees (solid blue columns) and in brood samples (hatched blue columns) by RT-qPCR were corrected using the bias value provided by the methods for ABPV, BQCV, SBV, and VDV1 quantification (A, B, E, and F, respectively). The dashed blue line on each graph indicates the thresholds between groups with low and high viral loads. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Among the samples containing DWV loads above 6 $\log_{10}$ genome copies/bee or larva (n = 19), 89% were collected from colonies with health issues. High VDV1 loads (above 7 $\log_{10}$ copies/bee or larva; n = 53) were detected in both colonies displaying no clinical signs (45%) and with health issue (55%). For both DWV and VDV1, the most frequently reported clinical signs were brood death or mosaic brood (53% and 52%, respectively), and colony depopulation (35% and 31%, respectively). The crippled bees were more frequently reported for DWV than for VDV1 (35% and 10%, respectively).

Most samples with SBV loads exceeding 9 $\log_{10}$ genome copies per bee (n = 23) had been collected from colonies with health disorders (96%). Colony depopulation had been reported (41%) as well as honeybee or brood death (or mosaic brood). Coloured larvae (or with a saccule) had been observed in four colonies (18%) and coloured capped cells in two colonies (moreover, BQCV genomes were also quantified in both samples at 3.83 and 4.36 $\log_{10}$ copies/bee).

4. Discussion

In this study we describe the performance of five TaqMan® RT-qPCR methods used to quantify the main honeybee viruses (ABPV, BQCV, DWV, SBV and VDV1) in naturally-infected samples collected in France between 2012 and 2019. CBPV was also quantified using the previously-validated RT-qPCR method (Blanchard et al., 2012). The distribution of viral load frequency was used to suggest a diagnostic threshold between covert and overt infections, as had been previously established for CBPV (Blanchard et al., 2007).

TaqMan® qPCRs were developed for virus quantification because this assay, which uses a probe in addition to the forward and reverse primers, provides more specific amplification than assays based on incorporating intercalating dye (i.e. SYBR® Green). However, for qPCRs using highly-specific primers, both qPCR methods could be effective for quantifying CBPV (Schurr et al., 2017). From the literature available,

we selected the TaqMan® RT-qPCR methods for validation due to their ability to detect all the viral strains circulating in France. Notably, we did not validate the qPCR described by Chantawannakul et al. (Chantawannakul et al., 2006) for DWV quantification because it did not detect several French strains (data not shown). However, the BQCV qPCR developed by these authors was highly efficient in detecting all the strains available in our collection (data not shown). An RT-qPCR method for SBV had previously been partially characterised (Blanchard et al., 2014). We completed the method's validation by testing the accuracy of the SBV loads it was used to quantify. When we started this study, there was unfortunately no TaqMan® qPCR available to quantify the main variants of DWV. Consequently, we designed new primers and probes to specifically amplify DWV or VDV1 in the VP3 coding sequence. This genome sequence was selected because it was frequently involved in genetic recombination between the two DWV variants (Dalmon et al., 2017; Ryabov et al., 2014).

Our assessment of RT-qPCR method performance indicated that four of the five viral quantification methods provided accurate results. The DWV RT-qPCR method was the most accurate (Fig. 2). Notably, we did not find any systematic error, which would have indicated a problem with trueness. The bias in quantitative results compared to the theoretical load of plasmid was calculated to be zero. The other method for quantifying ABPV, BQCV, SBV and VDV1 overestimated or underestimated the plasmid loads. These systematic errors were corrected in accordance with the recommendations of French standard U47-600 (AFNOR, 2015) for the development and validation of veterinary PCR. The RT-qPCR method for VDV1 quantification in honeybees is the least accurate. The method should be improved by designing new primers and probes, which means that databases will need to contain more VDV1 sequences in order to find primers increasing qPCR sensitivity and specificity. Currently, new genomes of DWV variants are available in databases and new RT-qPCRs have recently been suggested (Bradford et al., 2017; Kevill et al., 2017). Moreover, the results' validity ranges varied depending on the RT-qPCR method used. Plasmid loads quantified by the DWV RT-qPCR method were accurate within the 2.4–10.4 $\log_{10}$ copies/bee range. The ABPV RT-qPCR method provided accurate results in a narrow range between 6.4–10.4 $\log_{10}$ plasmid copies/bee. Below and above these limits of validity, viral loads can be calculated, but the quantitative results' accuracy was outside limits ($\pm$ 1.0 $\log_{10}$ copies/bee). In addition to the need for improvement in the VDV1 RT-qPCR method, the quantification of ABPV should also be improved in order to quantify lower viral loads with greater accuracy. Another means of improvement could be to quantify ABPV and related viruses (Kashmir bee virus and Israeli acute paralysis virus) in a single assay (Francis and Kryger, 2012).

The methods discussed above were used to quantify ABPV, BQCV, CBPV, DWV, SBV and VDV1 loads in naturally-infected adult honeybees or brood. According to the sample size (n = 10 honeybees or larvae), the minimum detectable viral prevalence in each colony was 26% (with 95% of confidence). The detection frequency for viral loads between 1.00–3.99 $\log_{10}$ genome copies/bee (the greatest viral load measured being 13.76 $\log_{10}$ CBPV genome copies/bee, quantified in honeybees collected in 2015) followed a bimodal distribution for ABPV, CBPV, DWV variants and SBV data. These bimodal distributions could be explained by some honeybees limiting the viral infection and others experiencing uncontrolled viral replication. For CBPV viral load distribution, the two groups were separated by a viral threshold, set at 8 $\log_{10}$ genome copies/bee. This limit is consistent with the previously diagnostic threshold that was suggested to separate covert and overt CBPV infections (Blanchard et al., 2007). In this study, we found CBPV loads above 8 $\log_{10}$ genome copies/bee exclusively in samples collected from colonies with clinical signs, notably adult honeybee death at the hive entrance and trembling or crawling bees (both clinical signs of chronic paralysis disease). Consequently, we suggested that such limit between the two viral load groups of a bimodal distribution might be used to distinguish between covert and overt infections for other viral infections. A previous study categorised the quantitative titres using the same threshold (set at 7 $\log_{10}$ copies) for several viruses (Amiri et al., 2015). In our study, the lowest threshold for ABPV loads was set at 5 $\log_{10}$ genome copies/bee. This could indicate that honeybees have a lower tolerance to ABPV replication. Moreover, previous authors have suggested that the *V. destructor* mite may increase the virulence of ABPV (de Miranda et al., 2010). This mite is currently a threat to all honeybee colonies in Europe except those on a few isolated islands (e.g. Finland's Åland islands or France's Ushant Island). Discovering honeybees with high ABPV loads in seemingly healthy colonies could be linked to good mite-infestation control by beekeepers. In this study, ABPV infections were suspected after noticing colony depopulation and *V. destructor* infestation.

The highest threshold was set at 9 $\log_{10}$ genome copies for SBV. This limit is lower than the limit previously suggested by Blanchard et al. (Blanchard et al., 2014) which was 10 $\log_{10}$ genome copies/bee. Nevertheless, their high viral load limit could reflect a higher tolerance to SBV replication. However, susceptibility to viral replication could be related to age (Bailey, 1969). Sacbrood disease was clearly linked to brood death, with fluid under the larvae's tegument containing large quantities of viruses (a maximum of 13.35 SBV genome copies/larvae were measured in this study). No obvious signs were reported for adult honeybees infected by SBV but they may have a decreased lifespan (Bailey and Fernando, 1972). The detection of one sample of adult honeybees from a colony displaying no clinical signs but with an SBV load quantified at 10.63 $\log_{10}$ genome copies/bee might also be explained by the difficulties faced by beekeepers in observing clinical signs associated with this viral disease

In our study, since the distribution of BQCV loads followed a normal distribution, the threshold was set at the limit (8 $\log_{10}$ genome copies), including 97.5% of the results (two standard deviations of the mean). Few colony disorders were suspected as being associated with BQCV infections (queen larvae death and coloured capped cells – Table 2). Nine brood samples were found to have viral loads above the threshold, and six of them (67%) were dead queen larvae. A synergetic interaction between *N. ceranae* and BQCV has previously been found (Doublet et al., 2015). Nevertheless, since nosemosis has low prevalence in France (Laurent et al., 2015), overt BQCV infections are probably rare.

Many studies have resulted in similar conclusions about the critical impact of the *V. destructor* mite on DWV or VDV1 replication in honeybees. The mite's introduction leads to the selection of certain viral strains (Martin et al., 2012; Mondet et al., 2014); the stimulation of virus replication in honeybees (Prisco et al., 2011; Wu et al., 2017); an increase in clinical signs (Zioni et al., 2011) and colony death. Clinical signs (brood death, crippled bees) can be overcome, however, by bees performing their hygienic activities. These activities could explain the discovery of apparently healthy colonies despite a high DWV variant loads (Table 2). Stressors other than the mite may also reduce honeybee tolerance: honeybee exposure to clothianidin (a neonicotinoid pesticide) increases the DWV load in honeybees exposed to both stressors (Di Prisco et al., 2013). Moreover, the lower discrepancy between both groups in the bimodal distribution of VDV1 compared to those of DWV (Fig. 3D and F), could be correlated with differences in replicating properties of both viruses, or it could be explained by the lower accuracy of the VDV1 RT-qPCR method. Indeed, the high uncertainty suggests that VDV1 loads in the range of 6–8 $\log_{10}$ copies/bee could be quantified above or below the threshold set at 7 $\log_{10}$ copies/bee (Fig. 2E). Our suggested thresholds for DWV and VDV1 are consistent with previous studies quantifying viral loads in asymptomatic and symptomatic honeybees at approximately 6–8 and 9–10 $\log_{10}$ genome copies/insect respectively (Highfield et al., 2009; Ryabov et al., 2014). In this study, we found that DWV variants quantified with viral loads above the thresholds were most frequently VDV1 or recombinant viruses sharing the same VP3 sequence. Except in one sample collected in South-West France in 2017, the DWV genome's VP3 coding sequence has been quantified since 2014 at lower loads than those of VDV1. This

Table 2
Frequency of viral loads exceeding the threshold in samples collected from colonies with or without health issues, and associated clinical signs.

Virus	Colonies displaying no clinical signs		Colonies with health disorders			Total number of tested samples
	Number of tested samples	Frequency of samples with viral load above the threshold	Number of tested samples	Frequency of samples with viral load above the threshold	Frequency of clinical signs or disorders reported for samples with viral loads above the threshold	
ABPV	97	6% (n = 6)	117	18% (n = 21)	38% depopulation (n = 8) 24% dead honeybees (n = 5) 38% *V. destructor* (n = 8)	214
BQCV	113	1% (n = 1)	119	9% (n = 11)	36% coloured capped cells (n = 4)	232
CBPV	105	0% (n = 0)	242	53% (n = 134)	90% dead honeybees (n = 121) 87% neurological signs (n = 116) 47% black bees (n = 63) 45% depopulation (n = 60)	347
DWV	99	2% (n = 2)	134	13% (n = 17)	53% brood troubles (n = 9) 35% depopulation (n = 6) 35% crippled bees (n = 6) 24% dead honeybees (n = 4) 47% *V. destructor* (n = 8)	233
VDV1	45	53% (n = 24)	47	62% (n = 29)	52% depopulation (n = 15) 31% brood troubles (n = 9) 10% crippled bees (n = 3) 10% dead honeybees (n = 3) 41% *V. destructor* (n = 12)	92
SBV	98	1% (n = 1)	102	22% (n = 22)	41% depopulation (n = 9) 32% dead honeybees (n = 7) 27% brood disorders (n = 6)	200

could be evidence of changes in DWV strains circulating in France, still under the influence of the *V. destructor* mite, which was first detected in France in the 1980s (Wilfert et al., 2016).

In conclusion, we characterised the accuracy of five TaqMan® RT-qPCRs in quantifying the main honeybee viruses detected in Europe. Although VDV1 and ABPV RT-qPCR accuracy ranges could be improved, the viral loads measured by these methods and by other viral RT-qPCRs can be compared to thresholds suggesting a break in the balance between honeybees and viruses. These quantitative methods are of particular interest for investigating the causes of colony disorders. The viral load thresholds could also be used to study the effects on honeybee health of co-exposure to viruses and other stressors.

Conflict of interest statement

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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References

AFNOR, 2015. NF U47-600, Part 2: Animal Health Analysis Methods - PCR - Part2: Requirements and Recommendations for the Development and the Validation of Veterinary PCR, Norme Françaises. AFNOR, pp. 1–51. www.afnor.fr.
Amiri, E., Meixner, M., Nielsen, S.L., Kryger, P., 2015. Four categories of viral infection describe the health status of honey bee colonies. PLoS One 10, e0140272.
Bailey, L., 1969. The multiplication and spread of sacbrood virus of bees. Ann. Appl. Biol. 63, 483–491.
Bailey, L., Fernando, E.F.W., 1972. Effects of sacbrood virus on adult honey-bees. Ann. Appl. Biol. 72, 27–35.
Bailey, L., Gibbs, A.J., Woods, R.D., 1963. Two viruses from adult honey bees (Apis mellifera Linnaeus). Virology 21, 390–395.
Bailey, L., Ball, B.V., Perry, J.N., 1983. Association of viruses with two protozoal pathogens of the honey bee. Ann. Appl. Biol. 103, 13–20.
Bakonyi, T., Grabensteiner, E., Kolodziejek, J., Rusvai, M., Topolska, G., Ritter, W., Nowotny, N., 2002. Phylogenetic analysis of acute bee paralysis virus strains. Appl. Environ. Microbiol. 68, 6446–6450.
Benaets, K., Van Geystelen, A., Cardoen, D., De Smet, L., de Graaf, D.C., Schoofs, L., Larmuseau, M.H., Brettell, L.E., Martin, S.J., Wenseleers, T., 2017. Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival. Proc. Biol. Sci. 284.
Benjeddou, M., Leat, N., Allsopp, M., Davison, S., 2001. Detection of acute bee paralysis virus and black queen cell virus from honeybees by reverse transcriptase pcr. Appl. Environ. Microbiol. 67, 2384–2387.
Blanchard, P., Ribiere, M., Celle, O., Lallemand, P., Schurr, F., Olivier, V., Iscache, A.L., Faucon, J.P., 2007. Evaluation of a real-time two-step RT-PCR assay for quantitation of chronic bee paralysis virus (CBPV) genome in experimentally-infected bee tissues and in life stages of a symptomatic colony. J. Virol. Methods 141, 7–13.
Blanchard, P., Regnault, J., Schurr, F., Dubois, E., Ribiere, M., 2012. Intra-laboratory validation of chronic bee paralysis virus quantitation using an accredited standardised real-time quantitative RT-PCR method. J. Virol. Methods 180, 26–31.
Blanchard, P., Guillot, S., Antunez, K., Koglberger, H., Kryger, P., de Miranda, J.R., Franco, S., Chauzat, M.P., Thiery, R., Ribiere, M., 2014. Development and validation of a real-time two-step RT-qPCR TaqMan((R)) assay for quantitation of Sacbrood virus (SBV) and its application to a field survey of symptomatic honey bee colonies. J. Virol. Methods 197, 7–13.
Bradford, E.L., Christie, C.R., Campbell, E.M., Bowman, A.S., 2017. A real-time PCR method for quantification of the total and major variant strains of the deformed wing virus. PLoS One 12, e0190017.
Brettell, L.E., Mordecai, G.J., Schroeder, D.C., Jones, I.M., da Silva, J.R., Vicente-Rubiano, M., Martin, S.J., 2017. A comparison of deformed wing virus in deformed and asymptomatic honey bees. Insects 8.
Chantawannakul, P., Ward, L., Boonham, N., Brown, M., 2006. A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in Varroa mites collected from a Thai honeybee (Apis mellifera) apiary. J. Invertebr. Pathol. 91, 69–73.
Dainat, B., Neumann, P., 2013. Clinical signs of deformed wing virus infection are predictive markers for honey bee colony losses. J. Invertebr. Pathol. 112, 278–280.
Dalmon, A., Desbiez, C., Coulon, M., Thomasson, M., Le Conte, Y., Alaux, C., Vallon, J., Moury, B., 2017. Evidence for positive selection and recombination hotspots in deformed wing virus (DWV). Sci. Rep. 7, 41045.
de Miranda, J.R., Genersch, E., 2010. Deformed wing virus. J. Invertebr. Pathol. 103 (Suppl. 1) S48-61.
de Miranda, J.R., Cordoni, G., Budge, G., 2010. The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. J. Invertebr. Pathol. 103 (Suppl. 1) S30-47.
de Miranda, J.R., Bailey, L., Ball, B.V., Blanchard, P., Budge, G.E., Chejanovsky, N., Chen, Y.P., Gauthier, L., Genersch, E., De Graaf, D.C., Ribière, M., Ryabov, E., De Smet, L., Van Der Steen, J.J.M., 2013. Standard methods for virus research in Apis mellifera. J. Apic. Res. 52.
de Miranda, J.R., Cornman, R.S., Evans, J.D., Semberg, E., Haddad, N., Neumann, P., Gauthier, L., 2015. Genome characterization, prevalence and distribution of a macula-like virus from Apis mellifera and Varroa destructor. Viruses 7, 3586–3602.
Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G., Pennacchio, F., 2013. Neonicotinoid clothianidin adversely affects insect immunity

and promotes replication of a viral pathogen in honey bees. Proc. Natl. Acad. Sci. U. S. A. 110, 18466–18471.
Doublet, V., Labarussias, M., de Miranda, J.R., Moritz, R.F., Paxton, R.J., 2015. Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. Environ. Microbiol. 17, 969–983.
European Union, 2017. Regulation (EU) 2017/625 of the European Parliament and the Council of 15 March 2017on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products. Off. J. Eur. Union L95/91-142.
Feinberg, M., 2007. Validation of analytical methods based on accuracy profiles. J. Chromatogr. A 1158, 174–183.
Francis, R.M., Kryger, P., 2012. Single assay detection of Acute bee paralysis virus, Kashmir bee virus, and Israeli acute paralysis virus. J. Apic. Sci. 56, 137–146.
Gauthier, L., Tentcheva, D., Tournairea, M., Dainat, B., Cousserans, F., Colin, M.E., Bergoin, M., 2007. Viral load estimation in asymptomatic honey bee colonies using the quantitative RT-PCR technique. Apidologie 38, 226–235.
Grabensteiner, E., Ritter, W., Carter, M.J., Davison, S., Pechhacker, H., Kolodziejek, J., Boecking, O., Derakhshifar, I., Moosbeckhofer, R., Licek, E., Nowotny, N., 2001. Sacbrood virus of the honeybee (Apis mellifera): rapid identification and phylogenetic analysis using reverse transcription-PCR. Clin. Diagn. Lab. Immunol. 8, 93–104.
Highfield, A.C., El Nagar, A., Mackinder, L.C., Noel, L.M., Hall, M.J., Martin, S.J., Schroeder, D.C., 2009. Deformed wing virus implicated in overwintering honeybee colony losses. Appl. Environ. Microbiol. 75, 7212–7220.
Kevill, J.L., Highfield, A., Mordecai, G.J., Martin, S.J., Schroeder, D.C., 2017. ABC assay: method development and application to quantify the role of three DWV master variants in overwinter colony losses of European honey bees. Viruses 9.
Laurent, M., Hendrikx, P., Ribière-Chabert, M., Chauzat, M.P., 2015. A pan-European epidemiological study on honeybee colony losses 2012-2014. In: Commission, E. (Ed.), EPILOBEE - 2012-2014 Version 2, (13th. January 2016). www.ec.europa.eu.
Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M., Nikaido, S., Schroeder, D.C., 2012. Global honey bee viral landscape altered by a parasitic mite. Science 336, 1304–1306.
McMenamin, A.J., Flenniken, M.L., 2018. Recently identified bee viruses and their impact on bee pollinators. Curr. Opin. Insect Sci. 26, 120–129.
Mondet, F., de Miranda, J.R., Kretzschmar, A., Le Conte, Y., Mercer, A.R., 2014. On the front line: quantitative virus dynamics in honeybee (Apis mellifera L.) colonies along a new expansion front of the parasite Varroa destructor. PLoS Pathog. 10, e1004323.
Prisco, G.D., Zhang, X., Pennacchio, F., Caprio, E., Li, J., Evans, J.D., Degrandi-Hoffman, G., Hamilton, M., Chen, Y.P., 2011. Dynamics of persistent and acute deformed wing virus infections in honey bees, Apis mellifera. Viruses 3, 2425–2441.
Ribiere, M., Olivier, V., Blanchard, P., 2010. Chronic bee paralysis: a disease and a virus like no other? J. Invertebr. Pathol. 103 (Suppl. 1), S120–131.
Ryabov, E.V., Wood, G.R., Fannon, J.M., Moore, J.D., Bull, J.C., Chandler, D., Mead, A., Burroughs, N., Evans, D.J., 2014. A virulent strain of deformed wing virus (DWV) of honeybees (Apis mellifera) prevails after Varroa destructor-mediated, or in vitro, transmission. PLoS Pathog. 10, e1004230.
Schurr, F., Cougoule, N., Riviere, M.P., Ribiere-Chabert, M., Achour, H., Adam, D., Castillo, C., de Graaf, D.C., Forsgren, E., Granato, A., Heinikainen, S., Jurovcikova, J., Kryger, P., Manson, C., Menard, M.F., Perennes, S., Schafer, M.O., Ibanez, E.S.M., Silva, J., Gajger, I.T., Tomkies, V., Toplak, I., Viry, A., Zdanska, D., Dubois, E., 2017. Trueness and precision of the real-time RT-PCR method for quantifying the chronic bee paralysis virus genome in bee homogenates evaluated by a comparative inter-laboratory study. J. Virol. Methods 248, 217–225.
Wilfert, L., Long, G., Leggett, H.C., Schmid-Hempel, P., Butlin, R., Martin, S.J., Boots, M., 2016. Deformed wing virus is a recent global epidemic in honeybees driven by Varroa mites. Science 351, 594–597.
Wu, Y., Dong, X., Kadowaki, T., 2017. Characterization of the copy number and variants of deformed wing virus (DWV) in the pairs of honey bee Pupa and infesting varroa destructor or Tropilaelaps mercedesae. Front. Microbiol. 8, 1558.
Zioni, N., Soroker, V., Chejanovsky, N., 2011. Replication of Varroa destructor virus 1 (VDV-1) and a Varroa destructor virus 1-deformed wing virus recombinant (VDV-1-DWV) in the head of the honey bee. Virology 417, 106–112.