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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

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ABSTRACT

The *Chronic bee paralysis virus* (CBPV) is the aetiological agent of chronic bee paralysis, a contagious disease associated with nervous disorders in adult honeybees leading to massive mortalities in front of the hives. Some of the clinical signs frequently reported, such as trembling, may be confused with intoxication syndromes. Therefore, laboratory diagnosis using real-time PCR to quantify CBPV loads is used to confirm disease. Clinical signs of chronic paralysis are usually associated with viral loads higher than 10^8 copies of CBPV genome copies per bee (8 $\log_{10}$ CBPV/bee). This threshold is used by the European Union Reference Laboratory for Bee Health to diagnose the disease. In 2015, the accuracy of measurements of three CBPV loads (5, 8 and 9 $\log_{10}$ CBPV/bee) was assessed through an inter-laboratory study. Twenty-one participants, including 16 European National Reference Laboratories, received 13 homogenates of CBPV-infected bees adjusted to the three loads. Participants were requested to use the method usually employed for routine diagnosis. The quantitative results (n = 270) were analysed according to international standards NF ISO 13528 (2015) and NF ISO 5725-2 (1994). The standard deviations of measurement reproducibility (S_R) were 0.83, 1.06 and 1.16 at viral loads 5, 8 and 9 $\log_{10}$

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CBPV/bee, respectively. The inter-laboratory confidence of viral quantification (+/− 1.96 S_R) at the diagnostic threshold (8 $\log_{10}$ CBPV/bee) was +/− 2.08 $\log_{10}$ CBPV/bee. These results highlight the need to take into account the confidence of measurements in epidemiological studies using results from different laboratories. Considering this confidence, viral loads over 6 $\log_{10}$ CBPV/bee may be considered to indicate probable cases of chronic paralysis.

1. Introduction

Chronic bee paralysis is a worldwide viral disease of adult bees associated with various clinical signs such as trembling and neurological disorders. This disease is a serious threat to apiculture and contributes to colony weakening, with significant bee losses and mortalities observed in front of the infected hives (Ribiere et al., 2010). The aetiological agent is the chronic bee paralysis virus (CBPV), a non-enveloped virus with a segmented RNA genome. The CBPV genome has been sequenced but this information did not allow for the virus to be assigned to any specific family (Olivier et al., 2008a). The virus can be transmitted horizontally (e.g. by the faeces of infected bees) or vertically (by the queen) (Amiri et al., 2014; Ribiere et al., 2007). The disease is currently diagnosed through quantification of the CBPV genome in symptomatic or dead bees by reverse-transcription and quantitative PCR (RT-qPCR) (Blanchard et al., 2007). A strong correlation between chronic paralysis and high viral loads (over 8 $\log_{10}$ of CBPV genome copies per bee [$\log_{10}$ CBPV/bee]) has been demonstrated, particularly in symptomatic bees (Blanchard et al., 2007). The replication of the virus in the integrative centres and sensory neuropiles could explain the nervous disorders observed in diseased honeybees (Olivier et al., 2008b). Viral loads of 10 $\log_{10}$ CBPV/bee or more have been correlated with abnormal mortality and clinical signs of paralysis such as trembling (Ribiere et al., 2010). This correlation between the viral load and clinical signs has been confirmed experimentally by infecting adult bees with CBPV (Chevin et al., 2012; Youssef et al., 2015). Visible symptoms such as trembling and crawling bees were observed in experimentally-infected bees at day 5 post-infection and the infected bees died by day 7 post-infection. All the investigated bees were positive for CBPV with a high viral load (over 12 $\log_{10}$ CBPV/bee). Such results were confirmed in a more recent study comparing the clinical signs and virus genome load in CBPV-infected bees and bees infected with purified RNA segments of CBPV (Youssef et al., 2015). Five to six days post-infection, the viral load was over 8 $\log_{10}$ CBPV/bee in both conditions. Quantifying virus in bee samples may be important for identifying the aetiological agent of adult honeybee mortalities. Currently, the clinical signs of chronic bee paralysis can be confused with those of other diseases or intoxication (Ribiere et al., 2010) or when several bee pathogens and pesticides are acting synergistically (Bacandritsos et al., 2010).

The ANSES Sophia Antipolis Laboratory has been appointed as the European Union Reference Laboratory (EURL) for bee health and Reference Laboratory of the World Organisation for Animal Health (OIE) for bee diseases. The main scope of its reference activities is not only to harmonise the diagnosis of bee diseases by ensuring that the National Reference Laboratories (NRLs) use EURL-validated methods, but also to guarantee the proper use of these diagnostic methods.

The RT-qPCR method for quantifying the number of CBPV genome copies per bee is one of such validated methods (Blanchard et al., 2012). This method is used to quantify the CBPV load between 4 to 10 $\log_{10}$ CBPV/bee with an accuracy of 0.5 $\log_{10}$ CBPV/bee. This method has been implemented in several European NRLs or official laboratories and is used notably to evaluate the prevalence of chronic bee paralysis in Europe (EPILOBEE Consortium et al., 2016).

In 2015, the EURL for honeybee health organized an inter-laboratory comparison (ILC) in order to assess the analytical ability of the participating laboratories to quantify CBPV genomic RNA in bees. The ILC was organised following the requirements of NF EN ISO/CEI 17043 (2015). This report presents a statistical analysis of performance for the quantitative data reported by the participating laboratories. The accuracy (trueness and precision combined) of the results was evaluated according to international standards (NF ISO 13528 (2015) and NF ISO 5725-2 (1994)).

2. Material and method

2.1. Healthy bees

Three hundred emerging honeybees were collected in healthy honeybee colonies originated from one of the ANSES laboratory apiaries (ANSES, Sophia Antipolis, France), during the autumn of 2014. These bees were found to be CBPV-negative or with a CBPV load below the quantification limit of the RT-qPCR method.

2.2. CBPV-infected bees

Dead bees and trembling bees were collected in front of a symptomatic colony from one of the ANSES laboratory apiaries (ANSES, Sophia Antipolis, France), during the spring of 2014. The samples were identified as Sophia-2014-H20 according to the harvesting location, the sampling date, and the hive number. The diagnosis of chronic bee paralysis virus disease was established by RT-qPCR (Blanchard et al., 2012) and the CBPV isolate was characterised by sequencing the amplicon (Beckman Coulter Genomics) obtained by a conventional PCR targeting the CBPV RNA polymerase coding sequence (Blanchard et al., 2008).

2.3. Inter-comparison samples

Healthy bees were crushed for negative bee homogenates. Bees infected by the CBPV isolate Sophia-2014-H20 were used as positive samples. Bees were crushed in a pH 7.0 phosphate buffer (1 ml per bee) using an Ultra-Turax tube drive. The bee homogenates were clarified by centrifugation according to a previously published protocol (Blanchard et al., 2007). The viral load in the bee homogenate was adjusted to three levels (5, 8 and 10 $\log_{10}$ CBPV/bee) by diluting the positive bee homogenates in negative bee homogenates. The bee homogenates were aliquoted in 0.5 ml (using 2 ml tubes with O-ring cap) and conserved at below −70 °C. Each participant (21 participants in all) received three samples of bee homogenate adjusted to 5 $\log_{10}$ CBPV/bee, five samples adjusted to 8 $\log_{10}$ CBPV/bee, and five samples adjusted to 10 $\log_{10}$ CBPV/bee. The samples were packed by the EURL in accordance with IATA packing instruction 650 (UN3373 − Biological Substance, Category B). The triple-layered parcel was composed of an outer cardboard box, an isotherm box containing the sample box and two ice packs. The parcel was shipped in March 2015 at ambient temperature by an approved transporter (Trans Medical Service). The parcel was delivered within the following three days except for one participant, who received the parcel six days later. The organiser, involved as a participant, received the comparative samples two days later.

Reference method for quantifying the CBPV genome

The reference method (used by the EURL, accredited as a testing laboratory according to international standard ISO 17025) was based on the viral RNA purification and TaqMan® two-step RT-qPCR. This method had been previously validated according to the French standard NF U47-600 (Blanchard et al., 2012). 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. The 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's, 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 duplicate, 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 of each forward and reverse primers (qCBPV9: 5′-CGC AAG TAC GCC TTG ATA AAG AAC −3′; qCBPV10: 5′- ACT ACT AGA AAC TCG TCG CTT CG − 3′), 200 nM of the qCBPV probe (5′- (6-Fam) TCA AGA ACG AGA CCA CCG CCA AGT TC (Tamra)-3′), 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. The thermal cycling conditions were 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. The results are expressed as the mean of the two replicates for each reaction.

2.4. Method implemented in the participant laboratories

A total of 21 laboratories participated in the ILC: 15 European NRLs (from Belgium, Croatia, Denmark, Finland, France, Germany, Hungary, Italy, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden and the United Kingdom), five French laboratories from an accredited network diagnosing chronic bee paralysis by RT-qPCR (EPILOBEE Consortium et al., 2016), and the Canadian NRL.

The available data concerning the implementation of the RT-qPCR method described by Blanchard et al. (2012) in the 21 participating laboratories are presented in supplementary Table S1. The laboratories used commercial kits from several suppliers for RNA purification, cDNA synthesis and real-time PCR. The RT-qPCR results were converted into $\log_{10}$ CBPV/bee taking into account the volume of bee homogenate tested, the volume of the elution buffer used for RNA recovery from the spin column, the volume of purified RNA used for cDNA synthesis and the volume of cDNA tested by real-time PCR in each NRL. All the NRLs participating in the ILC used the same primers (Blanchard et al., 2007) and the same batch of pAb2 clone (quantified by spectrometry at 260 nm) provided by the organiser. The pAb2 clone (recombinant plasmid with CBPV PCR-targeted sequence) was used to establish the calibration curves for quantification of the CBPV load in the range of 2.0 to 8.0 $\log_{10}$ copies per 5 µl (Blanchard et al., 2012).

2.5. Sequence analysis

RT-PCR products were purified by using the QIAquick PCR Purification kit (Qiagen) and direct sequencing of both strands was performed by Beckman (France). Sequence alignments and phylogenetic trees were calculated with the CLUSTAL X (version 1.81) analysis program (Thompson et al., 1997). Confidence values were determined by the bootstrapping method, as implemented in CLUSTAL X. Visualization of the phylogenetic trees was performed using TREEVIEW, version 1.6.6 (Page, 1996).

2.6. Sample homogeneity and stability

The homogeneity and stability tests were performed by the EURL. The homogeneity of ten randomly selected positive samples stored at below −70 °C (tested in replicate) was estimated by calculating the standard deviations (*Ss*) between samples according to the formula in Annex B of international standard NF ISO 13528 (2015). This *Ss* was expected to be about 0.5 $\log_{10}$ CBPV/bee or below.

To test the sample stability during shipment, each positive sample was packaged as previously described. One parcel was kept at a temperature of 23 °C for 72 h. A second parcel was exposed to temperatures that could possibly occur during the transport to the Canadian laboratory (72 h at 23 °C and 88 h at 10 °C). The samples were kept at below −70 °C before analysis to test the stability of CBPV RNA.

2.7. Statistical analyses

The participant's data on CBPV quantification and the method used

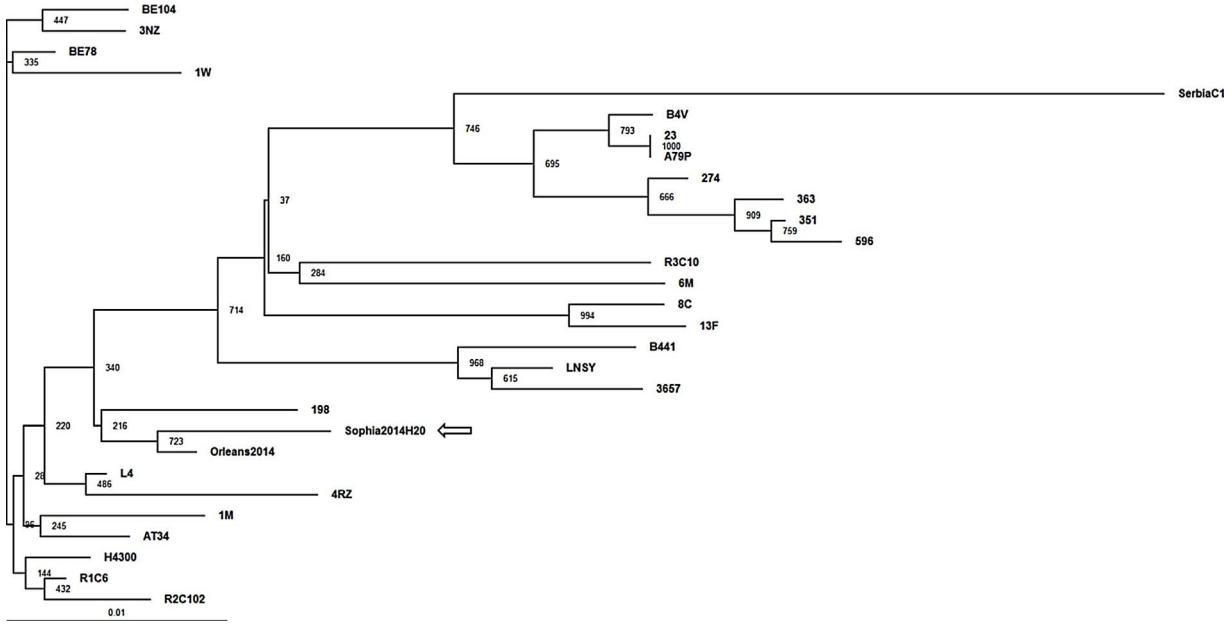


Fig. 1. Neighbour-joining phylogenetic tree constructed using 523 nt from published sequences of the CBPV RNA polymerase coding sequence (deposited in GenBank database) and the sequence of CBPV isolate Sophia-2014-H20 (Accession number: KX779523) selected for the inter-laboratory comparison (arrow). The sequences are identified by the CBPV isolate name (accession number provided in Supplementary Table S2). The phylogenetic tree was rooted using the CBPV isolates BE104 and 3NZ as outgroup. The numbers close to the nodes indicate the bootstrap values (in %; 1000 replicates). Bar: number of substitutions per site.

were registered and analysed by the EURL. The assigned values (robust mean: X^*, the robust standard deviation: S^*, the standard deviation of repeatability: S_r, and the standard deviation of reproducibility: S_R) of the viral loads (5, 8 or 9 $\log_{10}$ CBPV/bee) were calculated according to international standard NF ISO 13528 (2015) (using the A algorithm described in Appendix C) using the overall viral loads (x) found by the participants. The trueness of the participant's data was assessed using the statistical tests (z-score: $z = [x - X^*]/S^*$) also described in this international standard.

The mean standard deviation (S_{mean}) was calculated for each viral load using the standard deviations found by the participants for each sample (s), and the assessment of the precision (Mandel's k: $k = k'$ [s/S_{mean}] with the k' coefficient found in Mandel's k table at the 1% significance level) of the NRL's data was calculated according to international standard NF ISO 5725-2 (1994).

3. Result

3.1. Virus characterisation

The partial RNA-dependent RNA polymerase coding sequence of the Sophia-2014-H20 viral isolate was amplified and sequenced (GenBank accession number: KX779523). Fig. 1 shows that the isolate belongs to a phylum distant from the A-79P reference strains (GenBank accession number: EU122229.1). The sequence clustered in a lineage with a CBPV sequence obtained also in 2014 from another apiary located in France (isolate Orleans-2014, GenBank accession number: KX779524).

3.2. Sample homogeneity and stability

Before shipment, the Ss value was calculated for all the samples containing 5, 8, or 9 $\log_{10}$ CBPV/bee (Table 1). These values were within a range of 0.52 to 0.58 $\log_{10}$ and were higher than the expected value (0.5 $\log_{10}$ CBPV/bee). However, estimated Ss values were lower than the robust standard deviation S^*. Therefore, the samples were considered to be sufficiently homogeneous.

The stability of the samples after storage mimicking the shipment time and temperatures is shown in Table 1. Variations in the viral load were within the laboratory confidence range of the RT-qPCR estimated for the homogeneity test ($+/-$ 1.96 Ss). No significant reduction in the $\log_{10}$ CBPV/bee was found by RT-qPCR.

3.3. Sensitivity

Three samples containing 5 $\log_{10}$ CBPV/bee were analysed by the 21 participating laboratories (Fig. 2.a). One participant (coded O) did not detect CBPV in these samples. Taking into account the overall results (60 samples detected positive out of 63 tested samples), the diagnostic sensitivity was estimated to be about 95%. The samples with the highest viral loads (8 and 9 $\log_{10}$ CBPV/bee; Figs. 2.b and 2.c) were tested positive by all the participants (210 tested samples). The diagnostic sensitivity concerning samples with these highest viral loads was thus 100%.

3.4. Raw data and assigned values of the inter-laboratory comparison samples

The results of virus quantification obtained on the replicate analyses at the three viral loads were used to assess the accuracy of the participant's method (Fig. 2). For each viral load, the assigned values were the consensual values calculated using the overall participant data (Table 1). The only data excluded from the statistical analysis were the negative results obtained by participant O testing samples with the lowest viral load (5 $\log_{10}$ CBPV/Bee). The robust mean (X^*) calculated at the three viral loads (5.58, 8.02, and 9.24 $\log_{10}$ CBPV/bee) were in accordance with the expected values defined by the organiser (Table 1). Participant A, using the reference method described in this report, was one of the participants providing the most accurate results. No correlation was found between participant results and shipment time (three or six days for participants D, H, K, M, N, P and U), or the real-time PCR chemistry (participants Q and U used the SYBR-green chemistry while the others used the TaqMan chemistry), even with the use of single reaction RT-qPCR (participants E, F, G, K, N, P, Q, R, and T). The two components of accuracy are subsequently described as trueness and precision.

3.5. Assessment of the trueness of results

The values of the z-scores are shown in three histograms corresponding to the inter-laboratory comparison samples at the three viral loads (Fig. 3). Three participants provided unexpected results (participant codes D, T and O). As indicated previously, participant O found unexpected negative results when testing the samples containing 5.58 $\log_{10}$ CBPV/bee. At this lowest viral load, participants D and T found measurements statistically different from the assigned values ($p < 0.05$). The z-scores of participant D were below -3.0, indicating

Table 1
Homogeneity, stability, and assigned value of the inter-laboratory comparison samples. The results are expressed as $\log_{10}$ CBPV genome copies per bee. Homogeneity was evaluated using the aliquots of inter-laboratory comparison samples stored at below −70 °C. The both incubations mimicking the shipping conditions were used for the stability assessment. Robust statistics (algorithm A described in Appendix C of the NF ISO 13528 (2015) international standard) were used to calculate the assigned values of the inter-laboratory comparison samples at the three viral loads: 5, 8, and 9 $\log_{10}$ CBPV/bee.

Viral load ($\log_{10}$ CBPV/bee)	Homogeneity			Stability						Acceptance range[4]	Inter-laboratory comparison					
				72 h at 23 °C			72 h at 23 °C and 88 h at 10 °C				Assigned values					
	Mean	Ss[1]	n[2]	Mean	Variation[3]	n	Mean	Variation	n		X^*[5]	S^*[6]	S_r[7]	S_R[8]	U[9]	n
5	4.74	0.56	20	5.00	0.26	9	5.14	0.40	3	[3.64-5.84]	5.58	0.82	0.15	0.83	1.63	60
8	7.69	0.58	20	6.98	-0.71	13	7.29	-0.40	3	[6.55-8.33]	8.02	1.05	0.13	1.06	2.08	105
9	8.86	0.52	20	8.28	-0.58	13	8.71	-0.15	3	[7.84-9.88]	9.24	1.16	0.11	1.16	2.27	105

[1] Ss: standard deviation between samples.
[2] n: number of data.
[3] Variationn = stability mean − homogeneity mean.
[4] Acceptance range = homogeneity mean +/− 1.96 Ss.
[5] X^*: Robust mean.
[6] S^*: Robust standard deviation.
[7] S_r: repeatability standard deviation.
[8] S_R: reproducibility standard deviation.
[9] U: measurement uncertainty (1.96 S_R).

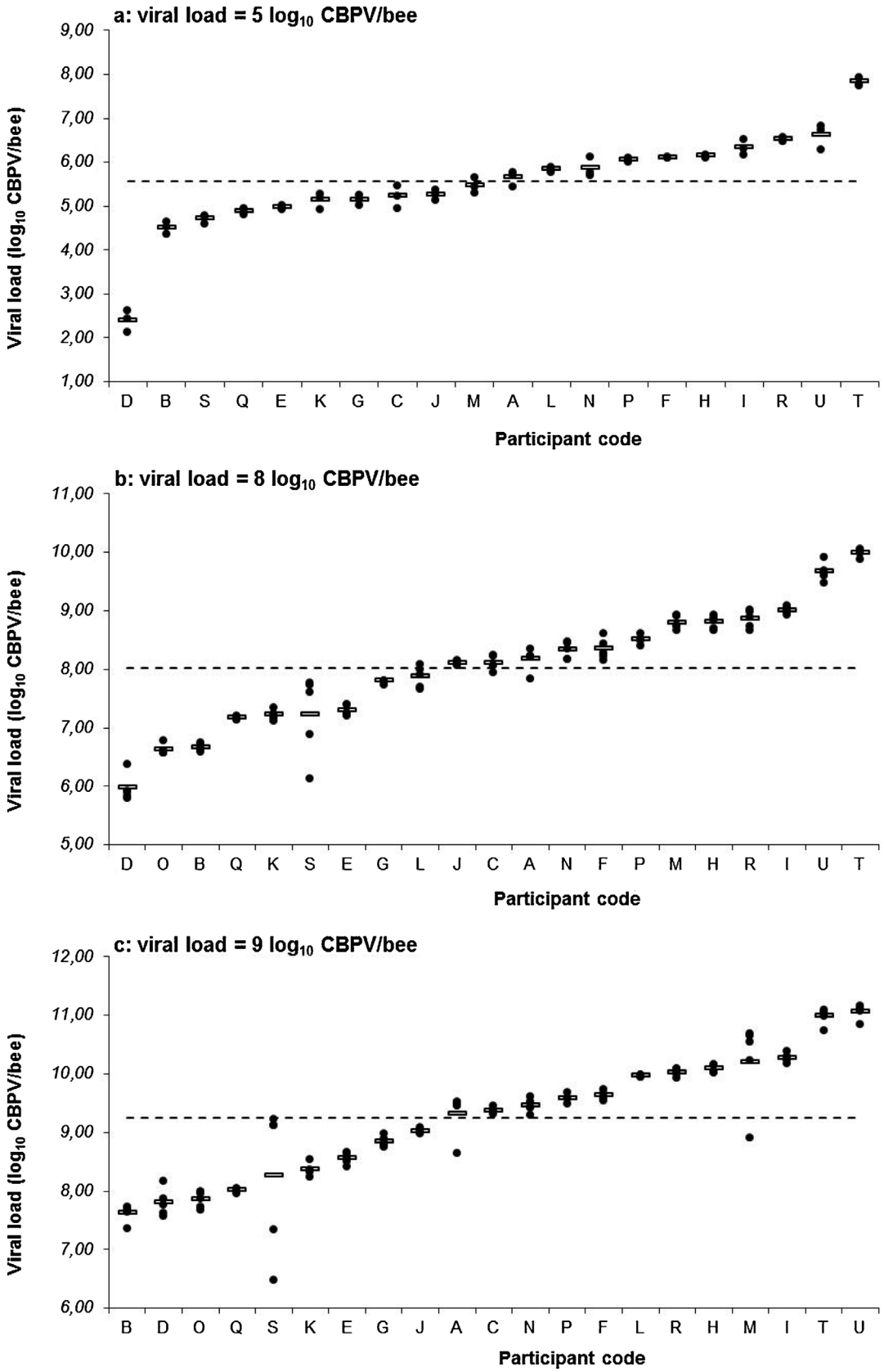
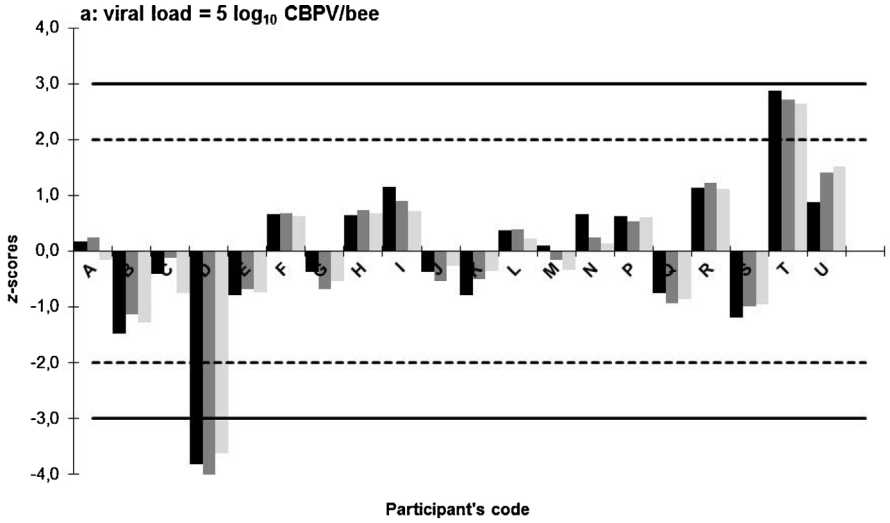
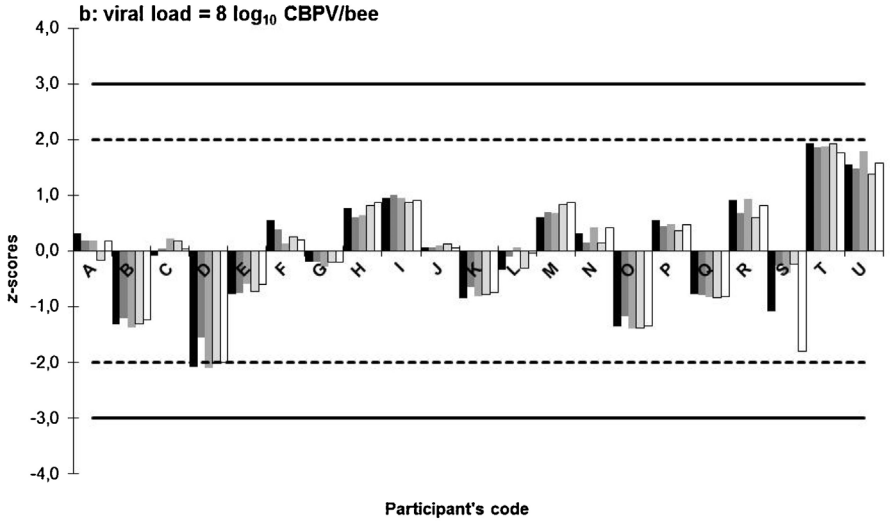


Fig. 2. Experimental results obtained by the participants (coded A to U) quantifying the CBPV in the inter-laboratory comparison samples. Three replicates containing 5 $\log_{10}$ CBPV/bee (Fig. 2.a) and five replicates containing 8 or 9 $\log_{10}$ CBPV/bee (Figs. 2.b and 2.c, respectively) were tested by each participant. The black bullets indicate the number of CBPV genome copies per bee ($\log_{10}$ CBPV/bee) quantified by RT-qPCR. The empty box is the participant's mean value. The dashed lines indicate the robust mean (X^*). The negative results found by participant O testing the samples adjusted to 5 $\log_{10}$ CBPV/bee are not shown in Fig. 2.a.





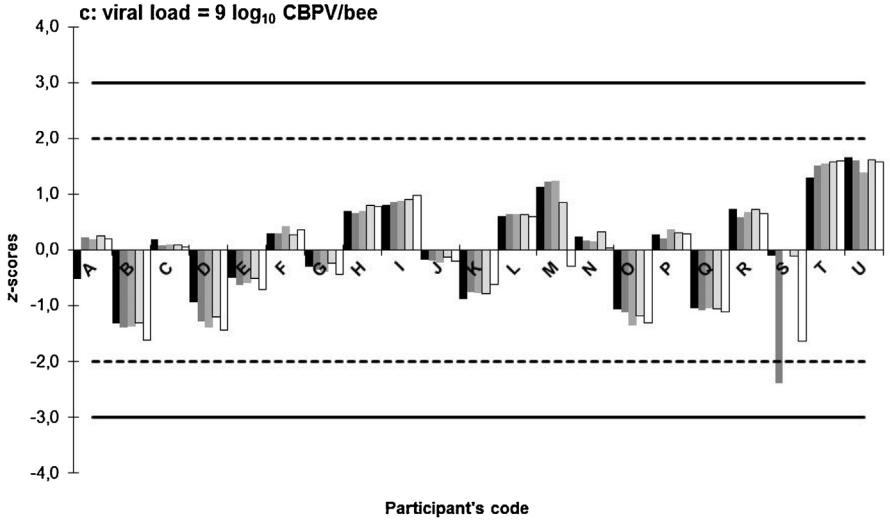


Fig. 3. z-scores calculated for each participant (coded A to U) quantifying the CBPV in the inter-laboratory comparison samples. The grey boxes indicate the individual z-score for the three replicates containing 5 $\log_{10}$ CBPV/bee (Fig. 3.a) and five replicates containing 8 or 9 $\log_{10}$ CBPV/bee (Figs. 3.c and 3.d respectively) tested by each participant. The dashed and solid lines indicate the limits of +/− 2 and +/− 3 respectively. For the viral load 5 $\log_{10}$ CBPV/bee, the negative results found by participant O are not taken into account.

that the participant's data was significantly lower than the assigned value and outside the 99% confidence range. The *z*-scores of participant T (between 2.0 and 3.0) indicated overestimations of the viral load outside the 95% confidence range.

3.6. Assessment of the precision of results

The *k* values were compared to an indicator derived from Mandel's statistical table at the 1% significance level. The participants' data were

usable because less than 25% of *k* values were above the critical threshold value. The *k* graphs (Fig. 4) reveal variability between replicate test results for participant M (at the viral load 9 $\log_{10}$ CBPV/bee) and participant S (at the viral loads 8 and 9 $\log_{10}$ CBPV/bee).

4. Discussion

An estimation of the viral load in biological samples may be used in the diagnosis and management of chronic diseases (Gullett and Nolte,

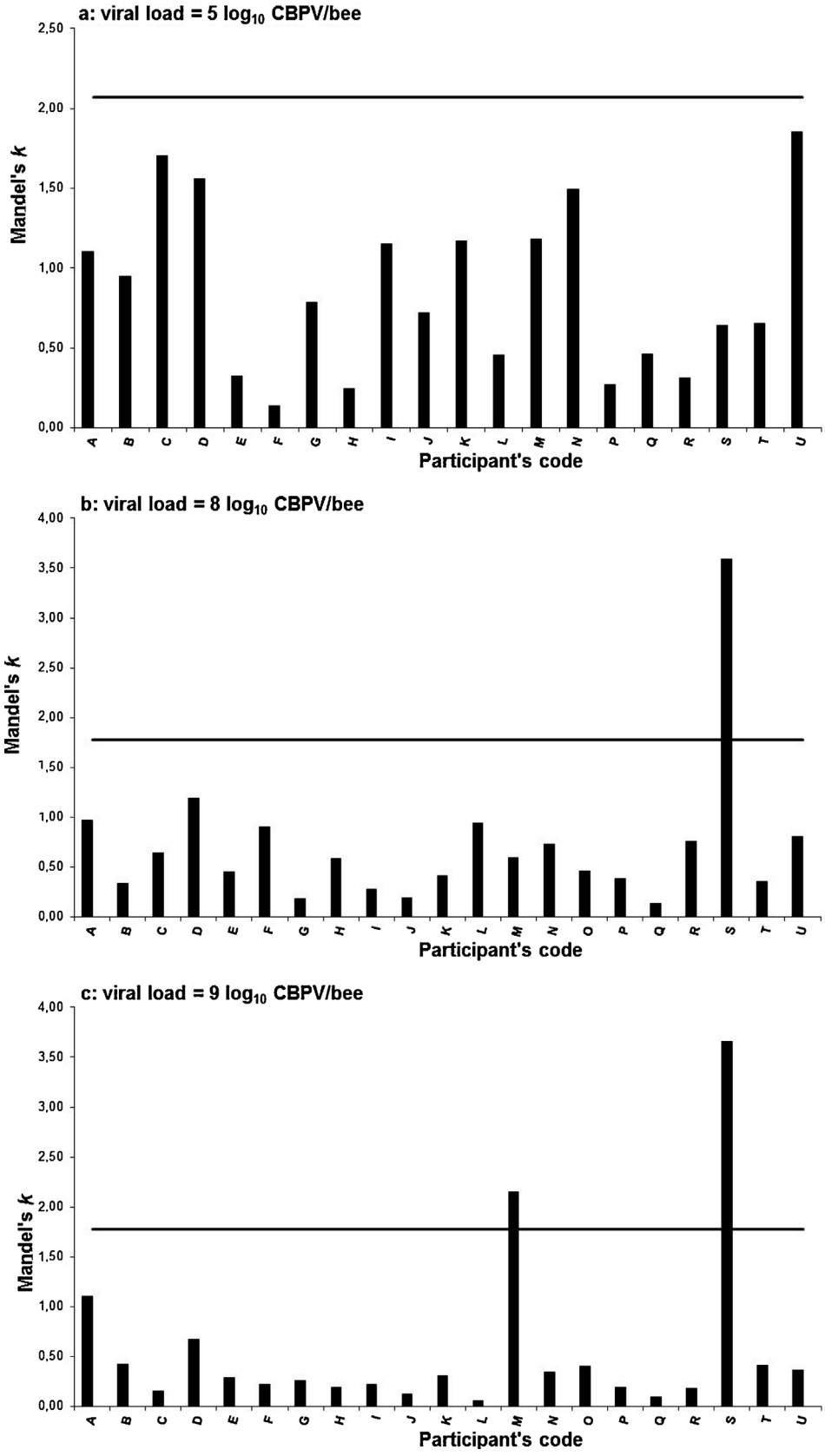


Fig. 4. Mandel's k calculated for each participant (coded A to U) quantifying the CBPV in the inter-laboratory comparison samples. The black boxes indicate the k value calculated for three replicates containing 5 $\log_{10}$ CBPV/bee (Fig. 4.a) and five replicates containing 8 or 9 $\log_{10}$ CBPV/bee (Figs. 4.b and 4.c) tested by each participant. The solid lines indicate the k' limit found in Mendel's k table at the 1% significance level. For the 5 $\log_{10}$ CBPV/bee level, the negative results found by participant O are not taken into account.

2015). Indeed, a variation in the number of viral genome copies reflects a breakdown in the balance between the parasite and its host, leading to the adverse effects of virus replication. Viral loads are also used to monitor effects of antiviral treatment. The accuracy of the measurement is of particular importance, especially when data provided by different laboratories are compared. A recent report by the European Commission compared the prevalence of chronic bee paralysis in Europe by analysing the CBPV load in symptomatic bees quantified by RT-qPCR in several laboratories (Laurent et al., 2015). The threshold for diagnosis of chronic bee paralysis was set at 8 $\log_{10}$ CBPV/bee by previous studies (Blanchard et al., 2007). The French data of CBPV loads in bees were collected by a network of accredited laboratories using the official method previously developed and validated by the EURL for honeybee health (Blanchard et al., 2012). Before performing viral diagnosis, these laboratories had adopted the method in accordance with the French standard NF U47-600-1 (2015). This standard describes the tests that should be performed using standard samples in order to guarantee the method. In other countries reporting quantitative data, the methods used to quantify CBPV were adapted from the official method without the preliminary tests described in the French standard. Consequently, the accuracy was not known for most of the laboratories prior to this study. Moreover, the accuracy strongly depends on controlling key points in the analytical process. The analytical process as a whole should be regulated in the laboratory's organisation in line with the international standard for diagnostic laboratories (NF EN ISO/CEI 17025, 2005) and recognised by an accreditation body. For example, the reference method for the quantification of CBPV has been validated and accredited for use in the EURL (Blanchard et al., 2012). The same approach can be used to manage the accuracy in intra-laboratory conditions while the sources of inter-laboratory variations must be estimated by comparing data obtained from several laboratories testing identical samples. In this report, we estimate trueness and precision (two components of accuracy) of the RT-qPCR for quantifying CBPV in bee homogenates by an ILC. The ILC was organised in keeping with international standard NF EN ISO/CEI 17043 (2015) in order to obtain reliable data.

The matrices of the inter-laboratory comparison samples were selected to minimise the variability in the results not directly related to the performance under evaluation. By more than 100 measurements of viral loads (in samples before and after storage in conditions mimicking the shipment), we showed that the number of CBPV genome copies in honeybee homogenate was both sufficiently homogeneous and stable. All the honeybee homogenates were prepared by the ILC organiser. Consequently, the analysis of performance of the method reported here did not cover variability related to upstream processing steps such as sampling and sample preparation. The laboratories diagnosing chronic bee paralysis currently take in account variability in the viral load of naturally infected bees by testing a pool of symptomatic honeybees (from 8 to 10 bees being recommended by the EURL). The performance of the methods evaluated in this report was influenced by all the steps of the process, from purification of the RNA to the qPCR reaction measured as a $\log_{10}$ number of CBPV genome copies per bee.

We also show that the detection sensitivity of CBPV by RT-qPCR at the lower viral load (5 $\log_{10}$ CBPV/bee) was about 95% (60 positive results out of 63 samples tested). Only one participant (participant O) did not detect CBPV genome at the lowest CBPV load which was selected to be about 100 fold over the reference method's limit of detection (Blanchard et al., 2012). This participant had received the samples within an appropriate delay (2 days later). One explanation of the negative results obtained by participant O was the use of an excessive internal positive control (supplementary Table S1) that could be in competition with the CBPV RNA (both targets being amplified during the same qPCR reaction). However, a limit of detection of about 5 $\log_{10}$ CBPV/bee should have a limited impact on the results expected when diagnosing the clinical disease because all the participants were able to detect the CBPV RNA in samples adjusted to 8 or 9 $\log_{10}$ CBPV/bee.

To assess the accuracy we assessed both trueness and precision. International standard NF ISO 5725-1 (1994) provides definitions of these performance criteria. Briefly, trueness is the closeness of agreement between the mean value obtained from a large series of test results and an accepted reference value. The measurement of trueness is usually expressed in terms of bias, which is the difference between the expected test results and an accepted reference value (Bias = x-X^*; with x = the observed result, and X^* = the reference value). Bias is the total systematic error. In our study, we calculated z-scores to assess bias in participant data instead of Mandel's h as described in the NF ISO 5725-2 (1994) standard. This choice was made because we also used the robust statistical analysis described in the NF ISO 13528 (2015) standard. The reference values were estimated without excluding data, while Mandel's h is calculated after excluding aberrant data identified by statistical tests (Cochran and Grubbs tests). The robust statistical analysis revealed the systematic errors of each participant's method. The real viral load in the samples was estimated in terms of consensual values (X^*). The latter were close to the mean values found by the EURL during the sample homogeneity and stability experiments (Table 1). Global bias was minimised by all participants using the same clone for generating the standard curves of the RT-qPCR methods. We considered that this is a crucial element in the harmonisation of methods to reduce systematic error. The stability of the samples and absence of variation in the results correlated with the shipment time exclude any systematic error arising from the study's organisation. At the estimated diagnostic threshold level (8 $\log_{10}$ CBPV/bee), five participants found mean values at least 1-$\log_{10}$ over (participants T and U) or under (participants B, D, and O) the robust mean (Fig. 2.b). However, no laboratory provided results significantly different from the assigned value no z-score < -2 or z-score > 2reported in Fig. 3.b). An alternative approach to reduce bias is suggested in the French standard NF U47-600-1 (2015), which describes the use of a reference material adjusted to the diagnostic threshold. The standard material should be calibrated by a reference laboratory or according to inter-laboratory testing. This reference material should be used systematically in the same RT-qPCR run so as to be able to compare the Cq (quantification cycle) values found with the reference material and the tested samples. Samples with a Cq-value lower than the Cq-value of the reference material can be considered as clinical case samples. In this ILC, the homogenate with the assigned value found at 8.02 $\log_{10}$ CBPV/bee could be considered the reference material (Table 1). However, for this purpose, very large amounts of reference material should have been prepared in order to satisfy the requirements of NRLs. This could be the aim of future reference material developments.

Another criterion evaluated in this study is each participating laboratory's precision of measurement. Precision is the closeness of agreement between independent test results obtained under stipulated conditions. It depends only on the distribution of random errors and does not relate to the true value of the specified value. Precision can be characterised by two components: repeatability and reproducibility. Repeatability is precision under the condition that test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time. Global repeatability is described in the present study as Sr (Table 1). Moreover, in this report, Mandel's k value provides an evaluation of the participant's repeatability compared to the repeatability mean found in the ILC (S_{mean}). Notably, the repeatability of viral loads found by participant S was significantly higher than the others at two viral loads (Figs. 4.b and 4.c). The high PCR efficiency (134%) found by participant S could be the cause of random errors. The accepted range of PCR efficiency was 75%-125% (NF U47-600-2, 2015). Accurate pipetting with regularly calibrated pipettes is critical in order to obtain accurate and precise data. Consistent pipetting of excess standard sample or pipetting of insufficient diluent in serial dilutions could increase PCR efficiency. Reproducibility is precision under the condition that test results are obtained with the same method on

identical test items in different laboratories by different operators using different equipment. S_R was used to estimate the inter-laboratory confidence of viral quantification ($+/-$ 1.96 S_R). For diagnostic viral load (8 $\log_{10}$ CBPV/bee), this confidence was estimated to be $+/-$ 2.08 $\log_{10}$ CBPV/bee. In the current quest to harmonise methods for quantifying CBPV in honeybees, viral loads between 6 and 8 $\log_{10}$ CBPV/bee found in symptomatic honeybees could be considered to indicate probable cases of viral chronic bee paralysis.

In conclusion, the international network of NRLs responsible for diagnosing chronic bee paralysis by RT-qPCR provided accurate results in this ILC. No significant systematic error (trueness) was found in the estimation of the number of genome copies in honeybee homogenates adjusted to the diagnostic threshold (8 $\log_{10}$ CBPV/bee). Only one laboratory had a lower precision (random error) than the others. However, the global uncertainty of measurements estimated in this study ($+/-$ 2.08 $\log_{10}$ CBPV/bee) should be taken into account in epidemiological studies on the prevalence of clinical chronic bee paralysis by interpreting the data from different laboratories. The accuracy could be improved by including a standard samples adjusted by the EURL to a diagnostic threshold.

Conflict of interest

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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2017.07.013.

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