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Intra-laboratory validation of chronic bee paralysis virus quantitation using an accredited standardised real-time quantitative RT-PCR method

Philippe Blanchard*, Julie Regnault, Frank Schurr, Eric Dubois, Magali Ribière

French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Sophia-Antipolis Laboratory, Bee Diseases Unit, BP 111, 06902 Sophia Antipolis, France

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A B S T R A C T

Chronic bee paralysis virus (CBPV) is responsible for chronic bee paralysis, an infectious and contagious disease in adult honey bees (*Apis mellifera* L.). A real-time RT-PCR assay to quantitate the CBPV load is now available. To propose this assay as a reference method, it was characterised further in an intra-laboratory study during which the reliability and the repeatability of results and the performance of the assay were confirmed. The qPCR assay alone and the whole quantitation method (from sample RNA extraction to analysis) were both assessed following the ISO/IEC 17025 standard and the recent XP U47-600 standard issued by the French Standards Institute. The performance of the qPCR assay and of the overall CBPV quantitation method were validated over a 6 log range from 10^2 to 10^8 with a detection limit of 50 and 100 CBPV RNA copies, respectively, and the protocol of the real-time RT-qPCR assay for CBPV quantitation was approved by the French Accreditation Committee.

1. Introduction

Chronic bee paralysis virus (CBPV) is known to be implicated in the weakening of honey bee colonies, inducing significant losses and mortality (Ball and Bailey, 1997; Ribiere et al., 2010a). The prevalence and distribution of CBPV infection are worldwide (Allen and Ball, 1996; Blanchard et al., 2009; Ribiere et al., 2008). Current diagnosis of the clinical disease is based on reverse-transcriptase PCR (RT-PCR) tests (Blanchard et al., 2008a; Ribiere et al., 2002) and a real-time quantitative RT-PCR (RT-qPCR) test (Blanchard et al., 2007; Celle et al., 2008). A strong correlation between chronic paralysis and high viral loads (>10^8 CBPV copies per bee) has been demonstrated, particularly in symptomatic bees (Blanchard et al., 2007). Standardisation of the RT-qPCR assay would be therefore useful for quantifying the CBPV genomic load in bee samples.

Significantly high mortality rates were observed in France during the 2007 beekeeping season. Bee samples from 50 apiaries from various parts of the country (23 departments) were analysed to evaluate the CBPV load. Of these apiaries, 62% showed high viral loads, exceeding 10^{10} viral genome copies per bee, confirming the diagnosis of the chronic paralysis and highlighting the major role of CBPV in bee mortalities (Blanchard et al., 2008b). A survey based on the detection of pathogens in diseased and dead honey bees observed in France in 2008 and 2009 clearly demonstrated that CBPV loads of 10^{10} copies or more per bee are correlated with abnormal mortality and paralysis symptoms such as trembling bees (Ribiere et al., 2010b). Furthermore, these data have been completed recently by a study on bee samples from various geographic regions where symptoms of chronic paralysis or abnormal mortality have been observed; 64% of these bee samples showed high CBPV loads, with over 10^{10} copies per bee (Blanchard et al., 2009). These results show that this RT-qPCR assay can be used to diagnose chronic paralysis associated with clinical symptoms observed in colonies.

To generalise this assay as the main criterion for diagnosis of CBPV, the reliability of results and the performance of the quantitation method must be demonstrated. However, full validation of molecular techniques, as per guidelines issued by the World Organization for Animal Health (OIE) or the French Standards Institute (AFNOR), and accreditation according to the ISO/IEC 17025 standard, is a relatively new concept, especially in the field of honey bee disease diagnosis. In France, the French Accreditation Committee (COFRAC) was designated in 2008 as the sole national accreditation body: accreditation is thus regarded as a public authority activity. Accreditation is a procedure that attests to a laboratory's technical competence and the reliability of its results. The Sophia-Antipolis Laboratory of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) has been designated as the National Reference Laboratory for bee diseases, Reference Laboratory of the OIE for bee diseases and, recently, European Union

* Corresponding author at: ANSES, Sophia-Antipolis Laboratory, Bee Diseases Unit, BP 111, Les Templiers, 06902 Sophia Antipolis, France.
Tel.: +33 0 492 948 769; fax: +33 0 492 943 701.
E-mail address: philippe.blanchard@anses.fr (P. Blanchard).

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Reference Laboratory for bee health. Therefore, the ANSES Sophia-Antipolis laboratory must guarantee that its results are obtained using valid methods and that its procedures comply with specific standards, such as the ISO/IEC 17025 standard, and are undertaken by competent and qualified staff.

The aim of this study was to validate the CBPV RT-qPCR method according to the XP U47-600 standard, "Requirements and recommendations for the implementation, the development and the validation of veterinary PCR for animal health analysis methods", as described by AFNOR. AFNOR protocols are first submitted to COFRAC for approval. Validation of the CBPV RT-qPCR protocol therefore involved a two-step procedure: first, the qPCR assay itself was evaluated in terms of (1) analytical specificity, (2) the PCR detection limit (DL_{PCR}) and (3) the PCR quantitation limit (QL_{PCR}) and finally, (4) the linearity and efficiency of the qPCR assay. Second, the entire protocol was evaluated in terms of (1) the method's detection limit (DL_{method}), (2) the diagnostic specificity and sensitivity on samples of known status and (3) the method's quantitation limit (QL_{method}) based on a validation range and accuracy profile. In each of the two steps, various parameters were determined, including measurement uncertainty (MU), deviations of repeatability and intermediate reliability.

2. Materials and methods

2.1. Real-time quantitative RT-PCR method for quantifying CBPV

Based on the TaqMan® two-step real-time quantitative RT-PCR (RT-qPCR) assay previously developed by Blanchard et al. (2007) and modified by Celle et al. (2008) on the Applied Biosystems 7500 System (Applera), the quantitation method evaluated in this paper was modified by integrating an exogenous internal positive control (TaqMan® IPC VIC™ Dye, Applied Biosystems), according to the manufacturer's recommendations, to reveal any false-negative samples. Briefly, the PCR reaction was performed in duplicate in a MicroAmp optical 96-well reaction plate, containing 1× Taqman® Universal PCR Master Mix with uracil-N-glycosylase (UNG) (2×, Applied Biosystems), 300 nM of each primer (qCBPV forward and reverse), 200 nM of the qCBPV probe, 1× Exo IPC Mic VIC (10×, Applied Biosystems), 1× Exo IPC DNA (50×, Applied Biosystems) and 5 μl of standard template (10^8–10^2 DNA copies) or cDNAs obtained as described previously (Ribiere et al., 2002) in a total 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 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Results are expressed as the mean of the two replicates of each reaction.

2.2. Analytical specificity

Analytical specificity must be assessed to ensure that the qPCR assay can (1) detect genetically variable viral isolates and (2) distinguish between the target analyte and other components genetically similar to the target and/or that may be present in the reaction and induce false positives. The analytical specificity must be assessed through *in silico* analysis and experimental tests. The *in silico* analysis was performed by a Blast search on the amplicon (101 bp) generated by the CBPV qPCR. The experimental specificity was evaluated by exclusivity and inclusivity tests. The exclusivity test was performed on cDNAs obtained from samples of other bee viruses, ABPV, SBV, BQCV and DWV. The inclusivity test was carried out on several CBPV strain isolates from various geographic regions, constituting a panel of samples that best represent CBPV genetic diversity.

2.3. Preparation of reference samples and experimental scheme to assess the detection and quantitation limits of the CBPV qPCR assay

Performance of the qPCR assay was evaluated in two steps: (1) determination of the detection limit and (2) determination of the quantitation limit. The detection and quantitation limits of the CBPV qPCR (DL_{PCR} and QL_{PCR}) were determined from the 3.82 kb plasmid obtained by cloning a 800 bp PCR fragment amplified from the putative viral RNA polymerase gene of CBPV into a pGEM®-T Easy vector (Promega). This plasmid was used for the DNA standard curve in Blanchard et al. (2007). The detection limit of the CBPV qPCR (DL_{PCR}) was determined from two-fold serial dilutions, the number of DNA copies ranging from 200 to 6.25 DNA copies for 5 μl of template. Three independent trials were performed on three independent dilution series, with eight replicates of each dilution level. The quantitation limit of CBPV qPCR (QL_{PCR}) was determined from 10-fold serial dilutions of the plasmid containing 0.2×10^8 to 0.2×10^3 DNA per μl in TE. A standard curve covering the range of 10^8–10^2 DNA copies per reaction was generated by analysing 5 μl of each dilution. Three independent trials were performed on three independent dilution series.

2.4. Diagnostic specificity and sensitivity

The specificity and the sensitivity of the method were assessed from bee samples experimentally infected by inoculation as previously described by Ribiere et al. (2000, 2002). Briefly, a 10^6-fold dilution of the purified virus was propagated by intra-thoracic route to naïve bees anaesthetised with carbon dioxide and obtained from a CBPV-free colony confirmed by CBPV RT-qPCR. Non-inoculated bees and bees inoculated with phosphate buffer were used as controls. The onset of visible symptoms of chronic paralysis (trembling bees, bees unable to fly and prostrate on the bottom of the cage) occurred on day 5 post-inoculation. Symptomatic bee samples and control bees were collected and tested with CBPV RT-qPCR to evaluate the specificity and the sensitivity of the method.

2.5. Preparation of reference samples and experimental scheme to assess the detection and quantitation limits of the CBPV quantitation protocol

Reference samples were obtained by loading a CBPV-negative homogenate of bees prepared as described previously (Blanchard et al., 2007) with purified CBPV virus prepared as described in Olivier et al. (2008). Based on the extracted RNA concentration determined by UV spectrometry and estimated at 350 ng/μl of purified CBPV virus, the conversion into RNA copy number was calculated using the molecular weight of both strands (1.98×10^6 g/mol) and the Avogadro number, resulting in a number of RNA copies equivalent to 1.06×10^{11} copies/μl. Working solutions were prepared in 200 μl and RNA was extracted using the High Pure Viral RNA Kit (Roche Diagnosis) according to the manufacturer's instructions. To assess the complete quantitation method, two steps were performed: determination of the detection limit and determination of the quantitation limit. The quantitation limit of the CBPV quantitation method (QL_{method}) was determined from 10-fold serial dilutions containing 1.6×10^9 RNA copies to 1.6×10^3 RNA copies in a volume of 200 μl. After RNA extraction and first-strand cDNA synthesis as described by Ribiere et al. (2002), the number of RNA copies ranged from 10^8 to 10^2 RNA copies for 5 μl of template used in CBPV qPCR. Three independent trials were performed on three independent dilution series, including

Table 1
Determination of the detection limit (DL_{PCR}) of CBPV RT-qPCR. Three trials were performed, each trial consisting of dilution series of the 3.82 kb plasmid at a load level ranging from 200 to 6.25 DNA copies in 5 μl of template. Eight replicates of each load level were tested. The smallest number of nucleic acid targets detected in at least 95% of the replicates of a given load level is shown in bold.

Copy number	Positive replicates trial 1	Positive replicates trial 2	Positive replicates trial 3	Total positive replicates	Total tested replicates	Frequency of detection
200	8	8	8	24	24	100.00%
100	8	8	8	24	24	100.00%
50	**8**	**8**	**8**	**24**	**24**	**100.00%**
25	6	5	5	16	24	66.67%
12.5	3	6	4	13	24	54.17%
6.25	4	3	4	11	24	45.83%

Table 2
Determination of the linearity range of CBPV qPCR. Three dilution series (S1–S3) were tested. The threshold cycle (C_T) measured for each amplification *vs.* the log copy number for each tested load level is given. Regression coefficients are also given.

	Load level (x)									
	100	1000	10,000	100,000	1,000,000	10,000,000	100,000,000	Slope (a)	Intercept (b)	Efficiency (%)
$\log_{10} x$	2	3	4	5	6	7	8			
S_1 C_T value	35.30	32.05	28.70	25.27	21.51	17.83	14.73	−3.48	42.44	93.91
S_2 C_T value	36.39	32.48	28.92	25.18	21.85	18.02	14.45	−3.64	43.51	88.37
S_3 C_T value	35.71	31.59	27.79	24.53	21.06	17.80	14.62	−3.49	42.15	93.6

two replicate RNA extractions for each dilution level. The detection limit of the CBPV quantitation method (DL_{method}) was determined from two-fold serial dilutions containing 1.28×10^4 RNA copies to 0.8×10^3 RNA copies in a volume of 200 μl. After RNA extraction and first-strand cDNA synthesis, the number of RNA copies ranged from 800 to 50 RNA copies for 5 μl of template used in the CBPV qPCR. Two independent trials were performed on two independent dilution series, including four replicates of RNA extractions for each dilution level. For both determinations, results are expressed as the mean of the two replicates of each reaction.

3. Results

3.1. Analytical specificity

Analytical specificity has been demonstrated in a previous study. CBPV specificity was confirmed by an *in silico* analysis (Blast search) and no significant similarity was found with other sequences in the database. Moreover, no amplification was detected in cDNAs obtained from ABPV, SBV, BQCV and DWV samples, attesting to exclusive specificity (Blanchard et al., 2007). Furthermore, a recent phylogenetic analysis conducted on bee samples from various geographical regions (Blanchard et al., 2009) demonstrated inclusive specificity, with the CBPV qPCR able to detect several genetically diverse isolates. Seven isolates were from France (including the two most divergent CBPV isolates known and described in Olivier et al., 2008) and 16 isolates were from other countries such as Austria, Poland, Hungary, Spain, Belgium, Denmark, Switzerland and Uruguay.

3.2. Determination of the detection limit of the CBPV qPCR

The detection limit of the CBPV qPCR (DL_{PCR}) is the smallest number of nucleic acid targets in a given template volume that was detected in at least 95% of the replicates.

Results are presented in Table 1. The DL_{PCR} of CBPV qPCR was determined to be 50 genome copies for 5 μl of template.

3.3. Determination of the linearity range and the quantitation limit of the CBPV qPCR

For each trial, a standard curve was generated by linear regression analysis of the threshold cycle (C_T) measured for each amplification *vs.* the $\log_{10}$ copy number for each standard dilution. The linear regression line is given by the formula $C_T = a\,[\log(x)] + b$, where a is the slope, b is the y-intercept, and x is the standard quantity. Table 2 shows the parameters for each linear regression model. Amplification efficiency (E) was calculated using the slope (a) of the linear regression for each standard curve using the formula $E = 10^{|1/a|} - 1$ (Table 2). Results obtained for the three trials showed high efficiency, ranging from 88 to 94%. To assess the linearity performance and determine the quantitation limit of the qPCR, the measured quantity for each series and each dilution level was determined in retrospect by using the formula: $\log x = [C_T - b]/a$. The obtained values were compared to the theoretical quantities and provided the mean bias, the standard deviation (SD) and the linearity uncertainty (U_{LINi}) for each dilution level. Following the AFNOR standard, the maximum allowed deviation for the measured quantities was set to $0.5 \log_{10}$, corresponding to a critical bias value ≤ 0.25 $\log_{10}$. As shown in Fig. 1, for each load level, the absolute value bias was less than the critical value, validating the linearity over the entire calibration range. According to the standard deviation of the measured quantity, the linearity uncertainty (U_{LINi}) was determined for each load level using the formula: $2[\sqrt{SD^2 + (x-y)^2}]$ where SD is the standard deviation of the measured values, x is the mean of the measured value and y is the theoretical value (Fig. 1). The combined linearity uncertainty (U_{LIN}) of the CBPV qPCR was given by the formula $U_{LIN} = |\sqrt{\sum U_{LINi}^2}/k|$

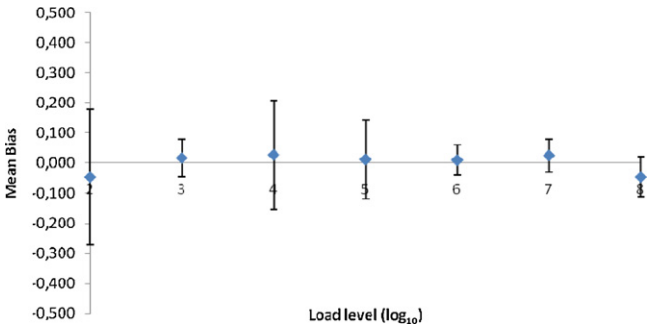

Fig. 1. Performance of linear regression for the CBPV qPCR. Mean bias was determined for each load level, bars represent the linearity uncertainty (U_{LINi}) given by the formula: $2[\sqrt{SD^2 + (x-y)^2}]$ where SD is the standard deviation of the measured values, x is the mean of the measured value and y is the theoretical value.

Table 3
Determination of the detection limit of the CBPV RT-PCR quantitation method (DL_{method}). Two trials were performed, each trial consisting of dilution series covering from 800 to 50 RNA copies in 5 μl of template. Four replicates of RNA extractions were tested. The smallest number of nucleic acid targets detected in all replicates of a given load level is shown in bold.

Copy number	Trial 1 positive replicates > LD_{PCR}	Trial 2 positive replicates > LD_{PCR}	Total positive replicates > LD_{PCR}	Total number of tested replicates	Frequency of detection
800	4	4	8	8	100.00%
400	4	4	8	8	100.00%
200	4	4	8	8	100.00%
100	**4**	**4**	**8**	**8**	**100.00%**
50	3	4	7	8	87.50%

Table 4
Diagnostic sensitivity and specificity. Diagnostic sensitivity (DSe) is given by the formula TP/(TP + FN), where TP and FN are, respectively, true positives and false negatives and are expressed as percentages. Diagnostic specificity (DSp) is given by the formula TN/(TN + FP) where TN and FP are, respectively, true negatives and false positives and are expressed as percentages.

	CBPV inoculated bees Known positive (5)	Control bees Known negative (19)
CBPV qPCR Results		
Positive	5 (TP)	1 (FP)
Negative	0 (FN)	18 (TN)
	DSe = 100%	DSp = 94.7%

where k is the number of dilution levels. U_{LIN} was determined to be 0.13 $\log_{10}$ in the range 2 $\log_{10}$ to 8 $\log_{10}$ CBPV copies for 5 μl of cDNA, with a quantitation limit of 10^2 genome copies.

3.4. Determination of the detection limit of the CBPV quantitation method

The study of the DL_{method} determines the amount of biological target that must be initially present in the sample to be detected. Results are presented in Table 3. The DL_{method} is the last dilution in which viral RNA can be detected in all replicates (100% frequency). The CBPV DL_{method} was determined to be 100 RNA copies for 5 μl of template volume.

3.5. Diagnostic specificity and sensitivity

The diagnostic specificity and sensitivity were assessed on samples from experimental infection. Results are presented as the percentage of positive samples found among the expected positive samples ($N = 5$) for the diagnostic sensitivity (DSe), and negative samples found among the expected negative samples ($N = 19$) for the diagnostic specificity (DSp) (Table 4). The positive samples showed high CBPV loads (over 10^{12} CBPV copies per bee), while one of the negative samples presented a low CBPV load of 10^4 copies per bee.

3.6. Determination of the quantitation limit of the CBPV quantitation method based on range validation and an accuracy profile

The assessment of a method's quantitation limit is based on the construction and interpretation of an accuracy profile to estimate the precision and reliability of the values. For each dilution series and each load level, the obtained quantities were compared to the theoretical quantity. Results are given in Table 5. Various parameters were determined to construct the accuracy profile, including the standard deviation of repeatability (SDrp), the standard deviation of reliability (SDrl) and the mean bias between the theoretical value and the mean of the obtained values.

To analyse the accuracy profile, the lower and upper tolerance interval limits of the complete CBPV quantitation method were determined using the following formula: mean bias $\pm 2\times$SDrl, and compared to the acceptability limits defined as $\pm 0.5 \log_{10}$ in the AFNOR standard (Fig. 2). The accuracy profile showed that the tolerance limits were within the acceptability limits in the 10^2–10^8 range, validating the CBPV quantitation method in this range with a quantitation limit of 100 CBPV copies for 5 μl of template, corresponding to the lowest tested dilution level.

4. Discussion

Molecular techniques such as real-time PCR are replacing conventional techniques and are widely used, including for detection of bee pathogens (Chen et al., 2005; Gauthier et al., 2007; Kukielka et al., 2008; Kukielka and Sanchez-Vizcaino, 2009; Siede et al., 2008; Blanchard et al., 2007). Validation of laboratory results is essential for molecular detection of pathogens and diagnosis of infectious

Table 5
Construction of the accuracy profile for the complete CBPV quantitation method. Three trials (T1 to T3) were performed, each trial consisting of dilution series with loads ranging from 10^8 to 10^2 RNA copies for 5 μl of template. Two replicates of each RNA extraction were tested. The mean for each load level was determined, as were the standard deviation of repeatability (SDrp), the standard deviation of reliability (SDrl), the coefficient of variation of intermediate reliability (CVirl) and the mean bias between the theoretical quantity and the mean measured quantities.

	Load level (x)						
	100	1000	10,000	100,000	1,000,000	10,000,000	100,000,000
$\log_{10} x$	2	3	4	5	6	7	8
T1 $\log_{10}$	2.07	3.14	4.24	5.09	6.03	6.99	8.01
	1.95	3.16	4.25	4.87	6.03	6.92	8.06
T2 $\log_{10}$	1.90	2.95	4.14	5.09	5.97	7.09	8.08
	2.07	3.12	3.90	5.11	5.98	6.99	8.01
T3 $\log_{10}$	1.92	2.82	4.05	4.94	5.88	6.95	7.92
	1.74	2.93	3.91	4.96	5.88	6.83	7.89
Mean	1.94	3.02	4.08	5.01	5.96	6.96	8.00
SDrp	0.112	0.083	0.115	0.089	0.003	0.068	0.036
SDrl	0.125	0.150	0.165	0.101	0.075	0.088	0.083
CVirl	6.26%	4.99%	4.12%	2.03%	1.25%	1.26%	1.04%
Mean Bias	−0.06	0.02	0.08	0.01	−0.04	−0.04	0.00

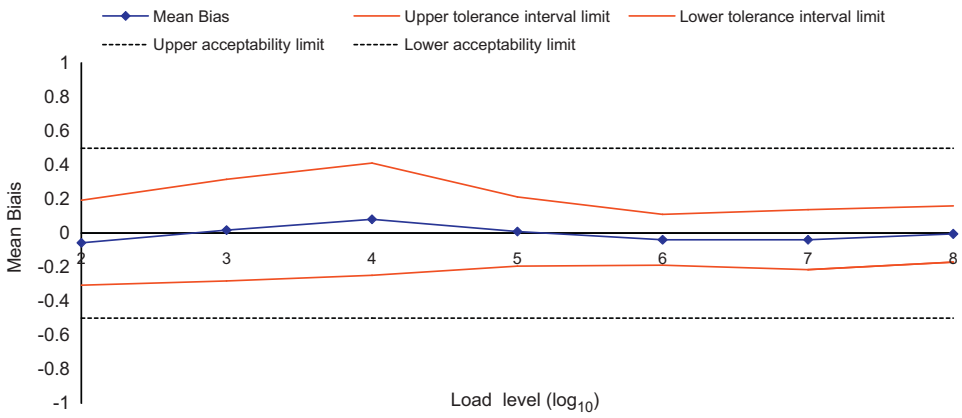

Fig. 2. Accuracy profile of the CBPV quantitation method. The acceptability limits were defined at $\pm 0.5\ \log_{10}$. The upper and lower tolerance interval limits were determined for each load level from the mean bias ± twice the standard deviation of reliability (SDrl).

bee diseases to ensure accurate, repeatable and reliable results. This paper describes the validation of a real-time quantitative RT-PCR method for the quantitation of chronic bee paralysis virus (CBPV), involving a two-step procedure: assessment of the qPCR assay (from cDNA to analysis result) and assessment of the entire quantitation method (from biological sample to analysis result). This process is based on the recent standard XP U47-600 developed by the French Standards Institute (AFNOR). This standard describes the requirements and recommendations for the implementation, development and validation of veterinary PCR in animal health, according to the NF EN ISO/IEC 17025 (NF, 2005) and to OIE recommendations (OIE, 2010). Intra-laboratory validation of the CBPV RT-qPCR assay according to the AFNOR standard is necessary to obtain accreditation from the French Accreditation Committee (COFRAC), thus recognising the laboratory's technical competence and reliability of results. This accreditation ensures the performance of the assay and makes it possible to propose this assay as a reference method for the detection and the quantitation of the CBPV virus.

The qPCR assay performances were assessed by determining its specific characteristics, such as analytical specificity, detection limit (DL_{PCR}), quantitation limit (QL_{PCR}) and linearity range. The analytical specificity of the CBPV qPCR has been demonstrated in previous studies: CBPV specificity has been confirmed *in silico* by a Blast search and exclusive CBPV specificity has been confirmed against other bee viruses, such as ABPV, SBV, BQCV and DWV (Blanchard et al., 2007). Moreover, a recent phylogenetic analysis conducted on bee samples originating from nine different countries in Europe and South America demonstrated inclusive CBPV specificity, the RT-qPCR CBPV quantitation method was able to detect genetically diverse isolates (Blanchard et al., 2009).

Detection and quantitation limits were evaluated from dilutions of the plasmid used to establish the standard curve. The detection limit of the CBPV qPCR in at least 95% of the replicates of each dilution series was determined to be 50 genome copies for a template volume of 5 μl of cDNA, corresponding to 4000 CBPV genome copies per bee. Samples containing fewer than 50 CBPV genome copies were considered CBPV negative. Based on the linear regression of standard curves, PCR efficiency proved to be high, ranging from 88 to 94%. The performance of linear regression was determined from the bias and the linearity uncertainty, obtained for each dilution level. Compared to the critical bias value of $\leq 0.25\ \log_{10}$ defined by the AFNOR standard, all absolute bias values were less than this value, validating qPCR linearity from $2\ \log_{10}$ to $8\ \log_{10}$ CBPV copies for 5 μl of cDNA. For this calibration range, the combined linearity uncertainty (U_{LIN}) — corresponding to measurement uncertainty includes both the bias of linearity and the reliability of the results, and was determined to be $0.13\ \log_{10}$. The quantitation limit was thus defined by the first load level, corresponding to 100 CBPV genome copies for a template volume of 5 μl of cDNA.

The performance of the complete quantitation method, from RNA extraction to the assay, was then assessed by determining specific characteristics such as the detection limit (DL_{method}), diagnostic sensitivity and specificity, quantitation limit (QL_{method}) and validation range of the accuracy profile. The detection and quantitation limits for the entire quantitation method were evaluated from CBPV-negative homogenate of bees spiked with a known quantity of purified CBPV virus. The detection limit of the CBPV RT-qPCR method was determined at 100 RNA CBPV copies, with a detection frequency of 100%. This estimated limit is not the absolute detection limit of the protocol, which was closer to the PCR detection limit (50 copies). The accuracy profile indicated the precision and the reliability of the protocol, and determined the validation range and the quantitation limit.

According to acceptability limits set at $0.5\ \log_{10}$, the tolerance limits of the CBPV quantitation method were within these limits over the whole calibration range (10^2–10^8), validating the method at a quantitation limit of 100 CBPV copies. Using this quantitation method, CBPV loads, ranging from $2\ \log_{10}$ to $8\ \log_{10}$ CBPV copies for a template volume of 5 μl of cDNA from bee homogenates, can be quantified with a measurement uncertainty evaluated at $0.13\ \log_{10}$, corresponding to a total quantity of 8×10^3 to 8×10^9 CBPV copies per bee.

The diagnostic sensitivity and specificity of the CBPV quantitation method were evaluated on bee samples from an experimental CBPV infection as described in Ribiere et al. (2002). Following inoculation with purified CBPV virus, visible symptoms such as trembling and weakened bees were observed at day 5 post-inoculation and all inoculated bees died by day 7 post-inoculation. All the investigated CBPV-inoculated bees ($N = 5$) tested positive for CBPV with a high CBPV load (over 10^{12} CBPV copies per bee), demonstrating a diagnostic sensitivity of 100%. Among the control bees (non-inoculated or phosphate buffer-inoculated, $N = 19$), for which no symptoms were recorded during the experiment, only one tested CBPV positive. However, this sample presented a low CBPV load of 10^4 copies per bee, likely related to a subclinical infection. In fact, CBPV can persist in healthy colonies at low CBPV loads; bees show none of the typical symptoms and the virus escapes detection by molecular methods (Ribiere et al., 2010b). Regardless of the source of the detected virus, this result indicates a diagnostic specificity of 94.7%.

This CBPV real-time quantitative RT-PCR method constitutes a substantial improvement for CBPV diagnosis, compared to the conventional methods used, such as the agarose gel immunodiffusion test (Ribiere et al., 2000) and the conventional PCR

assay previously developed in our laboratory (Ribiere et al., 2002) for which the detection limits were 10^9 and 10^4 CBPV copies, respectively (Blanchard et al., 2007).

Furthermore, sample quality and storage conditions must be considered in regard to the overall success of the RT-qPCR assay. For example, RNA integrity can greatly affect the detection of honey bee viruses (Chen et al., 2007; Dainat et al., 2011). Recommendations of the European Union Reference Laboratory in Sophia-Antipolis are (1) to collect preferentially symptomatic live bees or recently dead bees (dried or decomposing bees must be avoided), (2) to package bee samples in clean, sealed containers, such as cardboard packaging or paper, (3) to store bee samples at −20 °C and (4) to ship bee samples to the laboratory on dry ice or at low temperatures. Bee samples will be stored immediately upon receipt at −20 °C, before RNA extraction.

In conclusion, the performance of the CBPV real-time quantitative RT-PCR method described in this paper was validated according to the AFNOR XP U47-600 standard and approved by COFRAC. This method can thus be accepted as a reference method and proposed by our laboratory, as the European Union Reference Laboratory for bee health. However, to complete this validation, inter-laboratory proficiency tests should be carried out to evaluate the reproducibility of the method and its overall uncertainty.

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