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Standard operating procedure: Detection and quantification of the chronic bee paralysis virus (CBPV) by real-time RT-PCR

Sophia Antipolis Laboratory

National Reference Laboratory – Honey Bee Health

European Union Reference Laboratory – Honey Bee Health

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History of the method

A method can be updated in order to take changes into account.

A change is considered major when it involves the analytic process, the scope or critical points of the analysis method, the application of which may modify the performance characteristics of the method and/or the results. A major change requires major adaptations and either total or partial revalidation.

A change is considered minor if it provides useful or practical clarifications, reformulates the text to make it clearer or more accurate, or corrects minor errors. A minor change in the method does not alter its performance characteristics and does not require revalidation.

The table below summarises the version history of this method and provides qualifications for the changes.

Version	Nature of the changes	Date	Main changes
V00	Creation	November 1st, 2013	Initial version: protocol established according to the ANA-I1.MOA.13 (revision 02)
V01	Minor	February 20th, 2015	Protocol revised according to the ANA-I1.MOA.13 (revision 3)
V02	Minor	May 30th, 2018	- Editorial changes (according to ANSES' model) - Use of an alternative real-time PCR thermocycler - Clarification of several stages of the process - Quantitative results expressed in $\log_{10}$ of equivalent genome copies per volume or per honeybee

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Foreword

This method has been optimised by:

ANSES – Sophia Antipolis Laboratory

National Reference Laboratory for Honey Bee Health

European Reference Laboratory for Honey Bee Health

OIE Reference Laboratory for Honey Bee Diseases

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Warnings and safety precautions

The user of this method should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations.

All actions taken in accordance with this method must be performed by employees who have attended relevant training.

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1 Purpose and scope

This protocol describes the detection and quantification of the chronic bee paralysis virus (CBPV) by real-time RT-PCR (RT-qPCR) in honeybee samples. The method is based on the quantification of the RNA-dependent RNA polymerase (RdRp) gene sequence from the CBPV genome (Blanchard *et al*., 2007).

2 Principle of the method

Total RNA is purified from honeybees and used to produce cDNA by reverse transcription. The subsequent real-time PCR technique uses a TaqMan® probe (labelled with a reporter fluorophore at the 5' end and a quencher fluorophore at the 3' end) and a DNA polymerase with an exonuclease activity. During amplification, the 5'-3' exonuclease activity of the polymerase cleaves the probe hybridised to the target sequence. This releases the reporter from the quencher's influence, thereby increasing the fluorescence emitted by the reporter. The amplification cycle number (Ct = threshold cycle) corresponding to a significant increase of emitted fluorescence by the reporter is therefore inversely proportional to the number of copies of the amplified target sequence. The Ct value is compared to a calibration curve obtained from 10-fold serial dilutions of standard samples (recombinant plasmid with the RdRp gene sequence) in order to convert the result in equivalent CBPV genome copies.

This document describes the use of an internal positive control (with its own probe and primers) which is co-amplified during the real-time PCR (duplex amplification reaction). This control checks the absence of PCR inhibitors in the reaction, thus eliminating any false-negative results.

Detection and quantification of CBPV by RT-qPCR using bee samples can be divided into six steps (see Supplementary Figure S1):

1. Bee sample
2. Bee homogenisation
3. Total RNA purification from bee homogenate
4. Reverse-transcription of RNAs
5. Real-time PCR reaction
6. Results validation

3 Equipment, consumables and reagents

Warning[1]: **Trade names or supplier names may be mentioned in the description of the equipment and of the products required to implement this method. This information is provided for users of the method and does not mean that ANSES recommends the exclusive use of these products. Similar products may be used if it has been demonstrated that they achieve the same results.**

Note 1: The final user must verify the conformity of the implemented protocol with the expected criteria defined by the EURL for Honey Bee Health (see Supplementary Table S1). The EURL for Honey Bee Health can help the laboratory to verify its method conformity (contact: eurl.bee@anses.fr).

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Equipment	- Micropipettes (0.5-10 µl, 2-20 µl, 10-100 µl, 100-1000 µl) - Vortex - Ice tray - Manual tissue homogeniser (15 ml Potter) or mechanical tissue lyser (MM301/MM 400 lyser Qiagen or IKA ULTRA TURRAX Tube Drive) - Spin micro-centrifuge - 96-well plates centrifuge - micro-tube incubator - Real-time PCR system and a computer coupled to the machine (ABI Prism 7500 Real Time PCR or Quant Studio 5, Applied Biosystems)
Consumables	- Sterile 2 ml tubes and 1.5 ml microtubes (RNase-, DNase-free) - Sterile IKA Tube DT-20M - Optical 96-well reaction plate (and optical plate sealer film) (Applied Biosystems) - Sterile filter tips (RNase-, DNase-free) - Powder-free latex gloves
Reagents	- Pure RNase- and DNase-free water (H_2O) - Phosphate buffer 0.01 M pH 6.8 - TE: Tris 10 mM –EDTA 1 mM, pH 8 - High Pure Viral RNA Kit (Roche Diagnostics) - Random Hexamer (pdN6) (Invitrogen) (working solution: 0.3 µg/µl in Tris 10 mM - EDTA 1 mM, pH 8) - Super Script II Reverse Transcriptase Kit (Invitrogen) - RNaseOUT - Recombinant Ribonuclease Inhibitor (Invitrogen) - dNTP 10 mM mix – PCR Grade (Invitrogen) - Real-time PCR Master MIX (Applied Biosystems) - Specific CBPV primers: qCBPV9 and qCBPV10 (stock solution: 100 µM; working solution: 20 µM in H_2O) (Table 1) - Specific CBPV probe: CBPV 2 (stock and working solution: 50 µM in H_2O). It is recommended to protect the fluorescent probe from the light with a piece of aluminium foil. - Internal Positive Control MIX (if such a control is used) (Applied Biosystems) - Internal Positive Control DNA (if such a control is used) (Applied Biosystems) - External positive control - Standard samples (pAb2 clone for calibration curve): serial dilutions of the clone in TE ranging from 1.0 x 10^2 copies/5µl (G2) to 1.0 x 10^8 copies/5µl (G8)[2]

Note 2: The EURL for Honey Bee Health can provide standard sample pAb2 clone (G9 = 1.0 x 10^9 copies/5µl) to the laboratories performing the CBPV quantitative PCR method described in this protocol (contact: eurl.bee@anses.fr).

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Table 1: Primers and probe

Primers and probe	Sequence
qCBPV 9	5' - CGC AAG TAC GCC TTG ATA AAG AAC - 3'
qCBPV 10	5' - ACT ACT AGA AAC TCG TCG CTT CG - 3'
CBPV 2 probe	5' - (6-Fam) TCA AGA ACG AGA CCA CCG CCA AGT TC (Tamra) - 3'

4 Sampling and sample processing

4.1 Bee samples

At least eight symptomatic or dead bees must be harvested outside the hive. The observed symptoms are reported on a commemorative document. This clinical information is transmitted to the laboratory with the bee sample. The clinical signs that can be associated with chronic bee paralysis are listed in Table 2.

Table 2: Clinical signs of chronic bee paralysis (Ball and Bailey, 1997)

1	Trembling honeybees in front of the hive
2	Dead honeybees in front of the hive
3	Crawling honeybees
4	Black and/or hairless honeybees
5	Honeybees rejected by guards
6	Occupied flight board

4.2 Bee homogenisation

- 8 to 10 bees are crushed in 8 to 10 ml of ice cooled phosphate buffer 0.01 M pH 6.8 (1 ml per bee)
- Homogenize (manually: at least 20 grinding actions; mechanically: 3 min at 30 Hz with MM301 or MM400 lyser or 3 min power 9 with the Tube Drive)
- Maintained on ice
- Centrifuge 10 min at 8,000 x *g*
- Collect the supernatant
- Centrifuge 10 min at 8,000 x *g*
- Collect the supernatant (final homogenate)

4.3 RNA purification[3]

The extraction of total RNAs is performed using 200 µl of bee homogenate and denaturing solution (guanidine), and the RNAs are purified using spin colon according to the manufacturer's instruction. Used 200 µl of phosphate buffer 0.01 M pH 6.8 (or negative control provide with RNA purification kit) as negative control all along the process. Final RNA recovery is performed with 50 µl elution buffer.

The RNAs are kept in ice until to be quickly analysed.

Note 3: In order to reduce the risk of contamination between samples, the EURL recommends briefly centrifuging the tubes before opening them. In addition, we recommend handling the tubes carefully to prevent aerosol formation and contact with the inside of the tube-caps.

4.4 Reverse transcription (RT)

The cDNA synthesis is performed following two steps:

i. The random hexamer hybridisation (Table 3):

Table 3: Hexamer hybridization reaction

Reagent	Volume per reaction (Final: 13.5 µl)
pdN6 (0.3 µg/µl)	1.0 µl
RNA sample	12.5 µl

Incubate 2 min at 70°C and 5 min at 4°C.

ii. The reverse transcription reaction (Table 4):

Table 4: Reverse transcription reaction

Reagent	Final concentration	Volume per reaction (Final: 20 µl)
First Strand Buffer (5 X)	1 X	4.0
dNTP (10 mM)	500 µM	1.0
RNase OUT (40 U/µl)	20 U	0.5
Super Script II RT (200 U/µl)	200 U	1.0
RNA sample:pdN6 (hybridised)		13.5

Incubate 60 min at 42°C and 5 min at 95°C.

4.5 Preparation of the real-time PCR reaction mix[4]

Prepare the real-time PCR mix (Table 5). Briefly vortex the mixture and protect it with an aluminium foil before use.

Note 4: The cDNA samples, the standards samples (calibration curve), the negative and positive controls are analysed in duplicate.

Table 5: Real-time PCR reaction

Reagent	Final concentration	Volume per reaction (Final: 25 µl)
H_20	-	3.6
qPCR MIX (2 X)	1X	12.5
Primer qCBPV 9 (20 µM)	320 nM	0.4
Primer qCBPV 10 (20 µM)	320 nM	0.4
Probe CBPV 2 (50 µM)	200 nM	0.1
Int. Pos. Control MIX (10 X)	1X	2.5
Int. Pos. Control DNA (50 X)	1X	0.5
cDNA		5.0

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4.6 Preparation of plate containing the calibration standards and cDNA samples

- Thaw the calibration curve points (G2 to G8 standard samples). Vortex the tubes rapidly, centrifuge shortly and keep them on ice.
- Place the tubes containing the cDNA samples (from the RT reaction) on ice.
- Add 20 µl of the qPCR reaction mix to each well.
- Add 5 µl of each standard sample (G2 to G8) to the specified wells.
- Add 5 µl of UltraPure™ DNase/RNase-free distilled water to wells for negative control.
- Add 5 µl of the CBPV external positive control cDNA in the corresponding wells.
- Add 5 µl of each sample to be analysed in the corresponding wells.
- Centrifuge the samples rapidly (pulse for a few seconds up to a maximum of 800 × *g*).
- Place the samples in the machine and launch the run.

Incubation conditions:

Uracyl N-glycosylase cleavage:		50°C for 2 min
Polymerase activation:		95°C for 10 min
PCR (40 cycles)	Denaturation:	95°C for 15 sec
	Annealing/Extension:	60°C for 1 min

4.7 Data analysis

4.7.1 Validation of results

- Standard curve:

Report the settings for the analysis and standard curve to a results sheet:

- the threshold value determined by the software (Threshold);
- the slope (Slope) of the standard curve, which must be between -2.92 and -3.92;
- the Y intercept (Y-Inter), which is the expected C_t value for 1 copy;
- the R^2 (correlation coefficient) which must be greater than 0.97.

It is recommended to compare the Ct values for each calibration curve point with the Ct obtained in previous experiments with the same standard curve (prepared using the same G9 sample). A coefficient of variation (CV=Standard Deviation/Mean) of <5% for each point validates the results obtained for the calibration curve.

- External positive control:

This control allows the monitoring of the efficiency of RNA extraction, RT and qPCR:

- report the number of CBPV genome copies (in $\log_{10}$) found with the positive control in a control chart (ex. Shewhart control charts[5]);

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- check if amplification level of the external positive control does not vary in comparison to previous experiments.

If a high variation is observed, an analysis of possible causes and impact on final result should be undertaken.

Note 5: The EURL for Honey Bee Health can provide CV control table and Shewhart control chart (contact: eurl.bee@anses.fr).

- RT and qPCR negative controls:

Check that no amplification occurred in the wells corresponding to the qPCR negative control and the wells corresponding to the RT negative control. If amplification is detected in any of the negative controls, the RT and/or the PCR reactions are invalid and must be redone.

- Extraction negative controls:

Check that the results from the negative process control (Phosphate Buffer used as negative sample all along the process) are below 3.6 $\log_{10}$ equivalent copies per bee (Blanchard *et al*., 2012). If this is not the case, the complete analysis must be redone after complete decontamination of work areas and the centrifuge.

- Internal positive control:

This control allows checking that there are no PCR inhibitors in the reaction. Check that the Ct results obtained with this control are comparable in all wells, irrespective of the sample. However, if a sample shows high levels of CBPV, there will be competition between the two targets to the advantage of the majority CBPV target (hence the Ct value will be high or indeterminate). For all the other samples the Ct of the control must not vary.

If the Ct of the control is indeterminate or >35 for an unknown sample (there is a risk of presence of PCR inhibitors), repeat the real-time PCR using a diluted cDNA sample (1/10 or even 1/100)[6]. The C_t value of this diluted sample must be equal to the Ct in the other wells. If this is not the case, repeat the RNA extraction for this sample.

*Note 6: Take into accounts this dilution factor (1/10 or 1/100) for the transformation of raw positive results in CBPV genome copies per bee (see § 4.5.2). A negative result even from diluted cDNA sample should be interpreted as "**Inhibited sample**".*

- Unknown samples:

Positive sample: if both Ct values obtained for the duplicated cDNA samples have a standard deviation > 1, redo the real-time PCR analysis for this sample. The Ct mean is used as final result.

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4.7.2 Raw data conversion in CBPV genome copies per bee[7]

Equivalent viral load (genome copies) in the well is determined from the standard curve. The final result corresponding to the viral load present in a bee is obtained by adding to the raw results the conversion factor (taking into account the volume proportion used at each step: bee homogenisation, RNA extraction, RT and PCR). For this protocol, the factor is +1.9 (considering a 100% recovery rate at each step).

Note 7: The EURL recommends converting the results of quantitative PCR into log_{10} (ie. 150,000 copies/bee = 5.2 log_{10} copies/bee). Values are indicated with one digit number.

Example of coefficient estimation:

- Considering an initial load of 4.0 log_{10} genome copies per bee;
- 8 bees in 8 ml of homogenate (4.0 log_{10} genome copies/ml);
- equivalent to 3.3 log_{10} genome copies in 200 µl of homogenate sample used for RNA purification;
- 3.3 log_{10} genome copies recovered in 50 µl of total RNA (final elution);
- equivalent to 2.7 log_{10} genome copies in 12.5 µl of RNA sample used for cDNA synthesis;
- 2.7 log_{10} genome copies recovered in 20 µl of reverse transcription reaction (final volume);
- equivalent to 2.1 log_{10} genome copies in 5 µl of cDNA sample use in real-time PCR.

Conversion factor = 4.0 – 2.1 = +1.9 log_{10}

log_{10} genome copies per PCR reaction +1.9 = log_{10} genome copies per bee

4.7.3 Interpretation criteria

The results of quantitative PCR are expressed according to Table 6. The final result must take into account contextual data, particularly in the case of characteristic symptoms.

Table 6: Expression of quantitative PCR results and diagnosis interpretation

Result in log_{10} of equivalent viral genome copies per bee	Result reported[a]	Diagnosis interpretation
Undetermined	<LOD method	**CBPV not detected**
≤ 3.9	<LOQ method	**CBPV detected, not quantified**
>3.9 and <8.0	Number of CBPV genome copies per bee	**CBPV detected**
≥8.0 and < 10.0		The diagnosis conclusion must take into account the characteristic symptoms of the disease (Blanchard *et al*., 2007; Ribière *et al*., 2010 a and b) - Without symptoms, the diagnosis conclusion is: **Chronic bee paralysis probable**. - If the symptom 1 (trembling) is observed, alone or with another symptom, or if at least two of the symptoms 2 to 6 are observed (see Table 2), the diagnosis conclusion is: **Chronic bee paralysis declared**
≥ 10.0		**Chronic bee paralysis declared** (Blanchard *et al*., 2007; Ribière *et al*., 2010 a and b)

a: LOD: limit of detection; LOQ: limit of quantification

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4.8 Storage and disposal of samples after analysis

Samples storage:

- Samples (honeybee homogenates, purified RNAs) are stored for 1 year in an ultra-low freezer at ≤ -65°C;
- cDNAs (RT products) and standard samples (pAb2 clone: G9) are stored for 1 year in a freezer at ≤ -16°C .

Diluted standard samples (pAb2 clone: G2 to G8) are stored for 1 month in a freezer at ≤ -16°C (thawed no more than three times).

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6 Supplementary figure

The Figure S1 shows the successive steps of the complete method for the detection and quantitation of CBPV in bee sample.

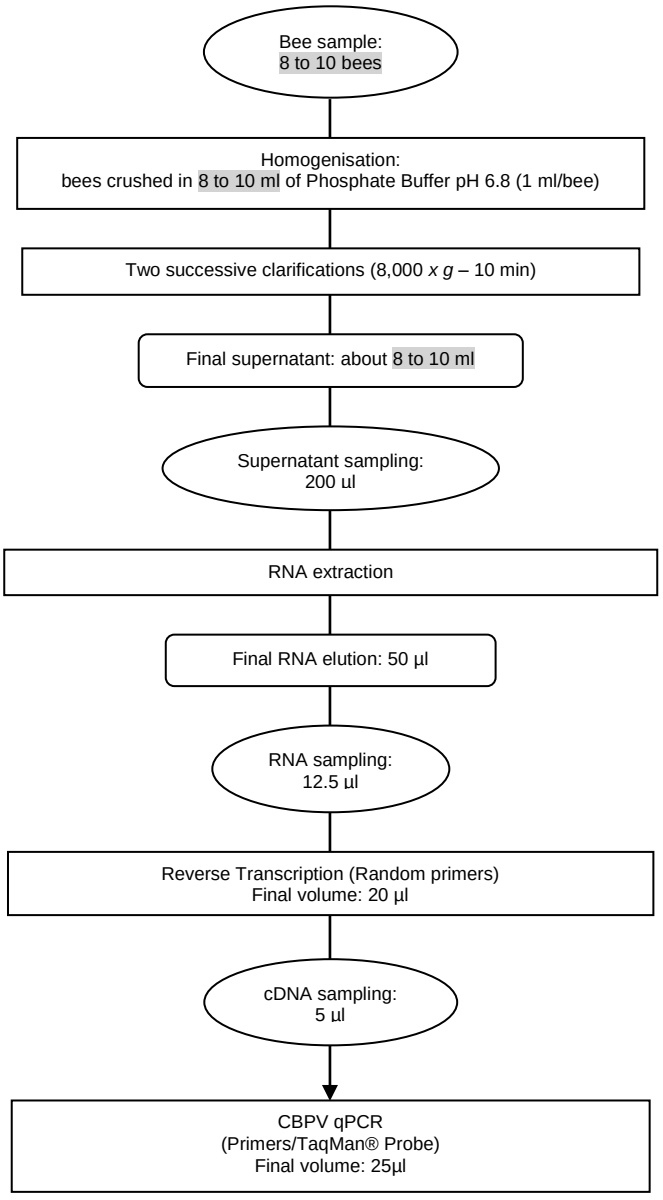

Figure S1: Synoptic of the quantitative PCR method

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7 Supplementary table

The Table S1 describes the performance criteria of the described method validated by the EURL for Honey Bee Health according to the norm NF U47-600 (www.afnor.fr).

Table S1: Validation criteria and RT-qPCR performances

Criteria	Expected result	Observed result
Analytical specificity	100%	100%
LOD qPCR (*minimum: 23 pos. samples / 24 tested samples*) (result expressed in $\log_{10}$ pAb2 copies / 5 µl)	≤ 4.0	1,7
Limit of quantitation (LOQ) qPCR (*Bias ≤ 0.25 $\log_{10}$*) (result expressed in $\log_{10}$ pAb2 copies / 5 µl)	≤ 6.0	2.0
Linearity range (*Bias ≤ 0.25 $\log_{10}$*) (result expressed in $\log_{10}$ pAb2copies / 5 µl)	4.0 to 8.0	2.0 to 8.0
qPCR efficiency	75 to 120%	96%
LOD Method (*minimum: 8 pos. samples / 8 tested samples*) (result expressed in $\log_{10}$ genome copies/bee)	≤ 6.0	3.9
LOQ Method (*Uncertainty ≤ 0.5 $\log_{10}$*) (result expressed in $\log_{10}$ genome copies/bee)	≤ 8.0	3.9
Accuracy range (*Uncertainty ≤ 0.5 $\log_{10}$*) (result expressed in $\log_{10}$ genome copies/bee)	8.0 to 10.0	4.0 to 10.0
Diagnosis sensitivity	≥ 90%	18/19 (95%)
Diagnosis specificity	≥ 90%	10/10 (100%)

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