Improvement of RT-PCR detection of chronic bee paralysis virus (CBPV) required by the description of genomic variability in French CBPV isolates.

Philippe Blanchard, Violaine Olivier, Anne-Laure Iscache, Olivier Celle, Frank Schurr, Perrine Lallemand, Magali Ribière

IMPROVEMENT OF RT-PCR DETECTION OF CHRONIC BEE PARALYSIS VIRUS (CBPV) REQUIRED BY THE DESCRIPTION OF GENOMIC VARIABILITY IN FRENCH CBPV ISOLATES.

Philippe Blanchard*, Violaine Olivier, Anne-Laure Iscache, Olivier Celle, Frank Schurr, Perrine Lallemand, Magali Ribière.

Agence Française de Sécurité Sanitaire des Aliments (AFSSA), Les Templiers, Route des Chappes, BP 111, 06902 Sophia Antipolis, France.

*Corresponding author

Mailing address: Blanchard Philippe, AFSSA Sophia-Antipolis, Unité Pathologie de l'Abeille, Les Templiers, BP 111, Fr – 06902 Sophia Antipolis

Telephone number: +33 (0) 492.943.726

Fax number: +33 (0) 492.943.701

e-mail: p.blanchard@afssa.fr

Abstract

A new RT-PCR test has been developed to diagnose Chronic bee paralysis virus (CBPV), able to detect genetically variable viral isolates. In fact, up to 8.7% divergence between partial nucleotide sequences from viral isolates from French honey bees was highlighted in a preliminary variability study. The previously-described RT-PCR was unable to detect all these viral isolates and RT-PCR diagnosis needed improvement. The new RT-PCR test allow to detect up to 40% of extra CBPV isolates.

Keywords: Chronic bee paralysis virus (CBPV), RT-PCR diagnosis, Genomic variability, *Apis mellifera.*

Short communication

Chronic paralysis is known to induce significant losses in honey bee colonies (Allen & Ball, 1996;Ball & Bailey, 1997). The pathology is characterized by clusters of trembling, flightless, crawling bees and by individual bees, sometimes hairless, standing at the hive entrance (Bailey *et al.*, 1983). Current diagnosis of the clinical disease is based on an Agarose Gel ImmunoDiffusion (AGID) test (Ball, 1999; Ribière *et al.*, 2000), complemented by RT-PCR (Ribière *et al.*, 2002). First isolated in 1963 (Bailey *et al.*, 1963), the aetiological agent of this pathology is the Chronic bee paralysis virus (CBPV), which consists of a single stranded RNA genome. Only a short fragment of the viral genome sequence has been reported to date (Ribière *et al.*, 2002) and research to sequence the complete CBPV genome is currently in progress in our laboratory.

Samples of trembling and crawling bees from apiaries in different French departments were recently subjected to AGID and RT-PCR tests. Although the AGID test gave positive results, no amplification was obtained with RT-PCR. The genomic variability between partial sequences of CBPV from several bee samples was indicated. Genomic variability in RNA viruses has been observed widely (Domingo *et al.*, 1996). This study led us to

48 design new RT-PCR primers able to detect all these viral samples and thereby improve
49 the CBPV diagnosis.
50 In this paper, we describe the development of a new RT-PCR diagnostic test for the
51 detection of CBPV isolates presenting genomic variability. The 5' RACE System
52 (Invitrogen) was used on the reference isolate (Isolate A) to obtain an additional
53 sequence from the one reported previously (Ribière *et al.*, 2002). Then, a preliminary
54 study of the genomic variability of seven CBPV viral isolates was begun. Bee samples
55 were collected from honey bee colonies presenting typical symptoms of chronic paralysis,
56 located in the French departments of Aisne, Alpes-Maritimes, Drôme, Essonne and
57 Rhône. Sample preparation, RNA extraction and cDNA synthesis were performed as
58 described previously (Ribière *et al.*, 2002; Blanchard *et al.*, 2007). Several primers were
59 tested and two sets of primers were retained, allowing to obtain by assembling a
60 sequence of 1285 bases for each isolate. Using these 2 sets of primers, a next PCR was
61 performed with a high-fidelity DNA polymerase (Platinum® *Pfx* DNA Polymerase,
62 Invitrogen). The PCR products were purified using the Qiaquick PCR purification kit
63 (Qiagen) and sequenced (Millegen, France). The 1285 bp sequence, located in the
64 putative viral RNA-dependent RNA polymerase (RdRp) gene region of CBPV, was
65 aligned using the Clustal W method in MegAlign in the Dnastar package.
66 Isolate A is the CBPV reference isolate obtained by infectivity test as previously
67 described (Ribière *et al.*, 2000). Isolate B is the sequence obtained from field samples
68 which presented the most variability with the reference isolate. Analysis of sequence pair
69 distances using the Clustal W method revealed 8.7% divergence between the two
70 sequences, mainly at the 3' region of this sequence whereas analysis of the amino acid
71 sequence revealed only 2.5% divergence between the two sequences. The alignment of
72 the sequence of the two isolates is illustrated in Figure 1.
73 These initial observations demonstrated the nucleotide sequence variability of the 3'
74 region, where primer pair CBPV 1 and CBPV 2 used for the first RT-PCR test was
75 designed (Ribière *et al.*, 2002), revealing 3 and 4 mismatches respectively in each

primer. A new RT-PCR test 2 using the primer pair designated CBPV 1-1 and CBPV 1-2 was designed by Primer3 Output and targeted the most highly conserved nucleotide region. The sequences, orientations and locations of these two oligonucleotide primer sets for the detection of CBPV by RT-PCR and the sizes of each amplification product are presented in Table 1 and in Figure 1. Amplifications were carried out in 25 µl total reaction volume using the Platinum® Taq DNA Polymerase (Invitrogen). The final PCR reaction contained 20 mM of Tris-HCl pH 8.4, 50 mM of KCl, 1.5 mM of MgCl2, 0.2 mM of dNTPs mix PCR grade, 0.4 µM of each primer, 2.5 units of Platinum® Taq DNA Polymerase and 2.5 µl of cDNA synthesised as described by Ribière *et al.* (2002). The thermal cycling conditions were 1 min at 95°C (denaturing of the template and activation of the enzyme), followed by 30 cycles consisting of denaturing at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, completed by a final elongation at 72°C for 5 min. Negative controls (RT and PCR controls) were included. The PCR products were then electrophoresed in 1% agarose gel in TAE buffer, stained with ethidium bromide, and visualised under UV light (Figure 2). No amplification was detected in the negative controls of either RT-PCR test (N_1 and N_2 for RT control and N'_1 and N'_2 for PCR control). Only the CBPV reference isolate and two of the seven tested isolates were detected with RT-PCR test 1 using primers CBPV 1 and CBPV 2, whereas all the isolates including isolate B (lane B_2) could be detected with RT-PCR test 2 using primers CBPV 1-1 and CBPV 1-2. These results confirm the performance of the RT-PCR test 2, which is able to detect viral isolates, even if they present genomic variability. The limit of detection of CBPV by this new RT-PCR test was of 10^4 CBPV copies per reaction which is identical to the one of the RT-PCR test 1, however lower than the limit of detection of the CBPV TaqMan PCR previously determined at 100 copies (Blanchard et al, 2007).

The genomic variability highlighted in this preliminary study of several French CBPV isolates showed 8.7% divergence between partial sequences obtained from these isolates and located in the putative viral RNA polymerase gene region. However, this

nucleotide sequence variability did not strongly affect the amino acid sequence of the transcribed RdRp protein. Sequencing of the complete CBPV genome sequence, consisting of two main positive sense single stranded RNAs (RNA 1 and RNA 2) (Overton *et al.*, 1982), is currently in progress in our laboratory. Preliminary results for genomic variability of these two RNAs confirmed the variability between isolates A and B, and revealed respectively 9.2 and 8.7% of divergence. CBPV detection by RT-PCR had to be improved to take this genomic variability into account. The RT-PCR test 2, described in this paper, is able to detect different viral isolates. To confirm these results, a complementary study was carried out to determine the proportion of isolates not detected by the RT-PCR test 1, allowing to assess the percentage of CBPV genomic variability. This study concerned 70 samples coming from France and European countries. All samples were detected CBPV positive with the second test (CBPV 1-1 / CBPV 1-2), while only 42 samples were detected CBPV positive with the first test (CBPV 1 / CBPV 2), indicating that nearly 40% of the samples are genetically divergent of the reference isolate. At the same time, a real-time two-step RT-PCR assay was developed, using a primers and probe set located in Figure 1 and able to detect and quantify such isolates (Blanchard *et al.*, 2007).

Genomic variability has already been described on isolates from different countries for honeybee viruses such as *Acute bee paralysis virus* (Bakonyi *et al.*, 2002) and *Sacbrood virus* (Grabensteiner *et al.*, 2001). We are currently carrying on the study of genomic variability of CBPV strains collected from different countries in Europe and worldwide. This study of isolates from several countries should validate the reliability of the RT-PCR test and sequencing of these isolates will permit phylogenetic analyses, assessing the genetic relationships between strains of different geographic origins.

Acknowledgements

This work was supported by the French Ministère de l'Agriculture et de l'Alimentation and with funds from the "Fonds Européens d'Orientation et de Garantie Agricole" (FEOGA), in

132 accordance with the French programme for the improvement of the production and
133 commercialisation of beekeeping products. We are grateful to the collaborators and
134 beekeepers for having kindly provided bee samples.
135

136 **Captions to figures**

137 Figure 1.

138 Alignment of the nucleotide sequences (1285b) of the two CBPV isolates: isolate A is the
139 reference isolate and isolate B, the one presenting the most variability. Nucleotides
140 differing between the sequences are indicated in white. The two primer sets used in RT-
141 PCR tests (CBPV 1 and CBPV 2 for RT-PCR test 1 and CBPV 1-1 and CBPV 1-2 for RT-
142 PCR test 2) and the primers (qCBPV forward, qCBPV reverse) and probe (qCBPV probe)
143 set used in quantitative real-time PCR described by Blanchard et al. (2007) are located.
144

145 Table 1.

146 Primers used for CBPV RT-PCR tests, CBPV 1 and CBPV 2 for RT-PCR test 1 (Ribière
147 *et al.*, 2002), CBPV 1-1 and CBPV 1-2 for RT-PCR test 2 (this article).
148

149 Figure 2.

150 Visualisation of the PCR products obtained with the RT-PCR test 1 (Ribière *et al.*, 2002)
151 and RT-PCR test 2, amplifying PCR fragments of 455 bp and 570 bp respectively.
152 Lane MW: 100 bp DNA Ladder (Invitrogen).
153 Lane N: RT negative controls.
154 Lane A: CBPV reference isolate A.
155 Lane B to lane H: CBPV isolates from different departments.
156 Lane N': PCR negative controls.

157 References

158

159 Allen, M. & Ball, B. V., 1996. The incidence and world distribution of honey bee viruses.
160 Bee World 77, 141-162.

161 Bailey, L., Ball, B. V. & Perry, J. N., 1983. Honeybee paralysis: its natural spread and its
162 diminished incidence in England and Wales. J. Apic. Res. 22, 191-195.
163 Bailey, L., Gibbs, A. J. & Woods, R. D., 1963. Two viruses from adult honey bees (*Apis*
164 *mellifera* Linnaeus). Virol. 21, 390-395.

165 Bakonyi, T., Grabensteiner, E., Kolodziejek, J., Rusvai, M., Topolska, G., Ritter, W. &
166 Nowotny, N., 2002. Phylogenetic analysis of acute bee paralysis virus strains.
167 Appl. Environ. Microbiol. 68, 6446-6450.

168 Ball, B. V., 1999. Paralysis. In *Bee disease diagnosis,* pp. 81-89. Edited by M. E. Colin, B.
169 V. Ball & M. Kilani: Options Mediterannéennes.

170 Ball, B. V. & Bailey, L., 1997. Viruses. In *Honey bee pests, predators, & diseases*, Third
171 edition, pp. 11-32. Edited by R. A. Morse & K. Flottum. Medina: A.I. Root
172 Company.

173 Blanchard, P., Ribière, M., Celle, O., Lallemand, P., Schurr, F., Olivier, V., Iscache, A. L.
174 & Faucon, J. P., 2007. Evaluation of a real-time two step RT-PCR assay for
175 quantitation of Chronic bee paralysis virus (CBPV) genome in experimentally-
176 infected bee tissues and in life stages of a symptomatic colony. J. Virol. Methods
177 141, 7-13.

178 Domingo, E., Escarmis, C., Sevilla, N., Moya, A., Santiago, F., Quer, E. J., Novella, I. S.,
179 Holland, J.J., 1996. Basic concepts in RNA virus evolution. The FASEB Journal
180 10, 859-864.

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.

Overton, H. A., Buck, K. W., Bailey, L. & Ball, B. V., 1982. Relationships between the RNA components of chronic bee-paralysis virus and those of chronic bee-paralysis virus associate. J. Gen. Virol. 63, 171-179.

Ribière, M., Faucon, J. P. & Pépin, M., 2000. Detection of chronic bee paralysis virus infection: application to a field survey. Apidologie 31, 567-577.

Ribière, M., Triboulot, C., Mathieu, L., Aurières, C., Faucon, J. P. & Pépin, M., 2002. Molecular diagnosis of chronic bee paralysis virus infection. Apidologie 33, 339-351.

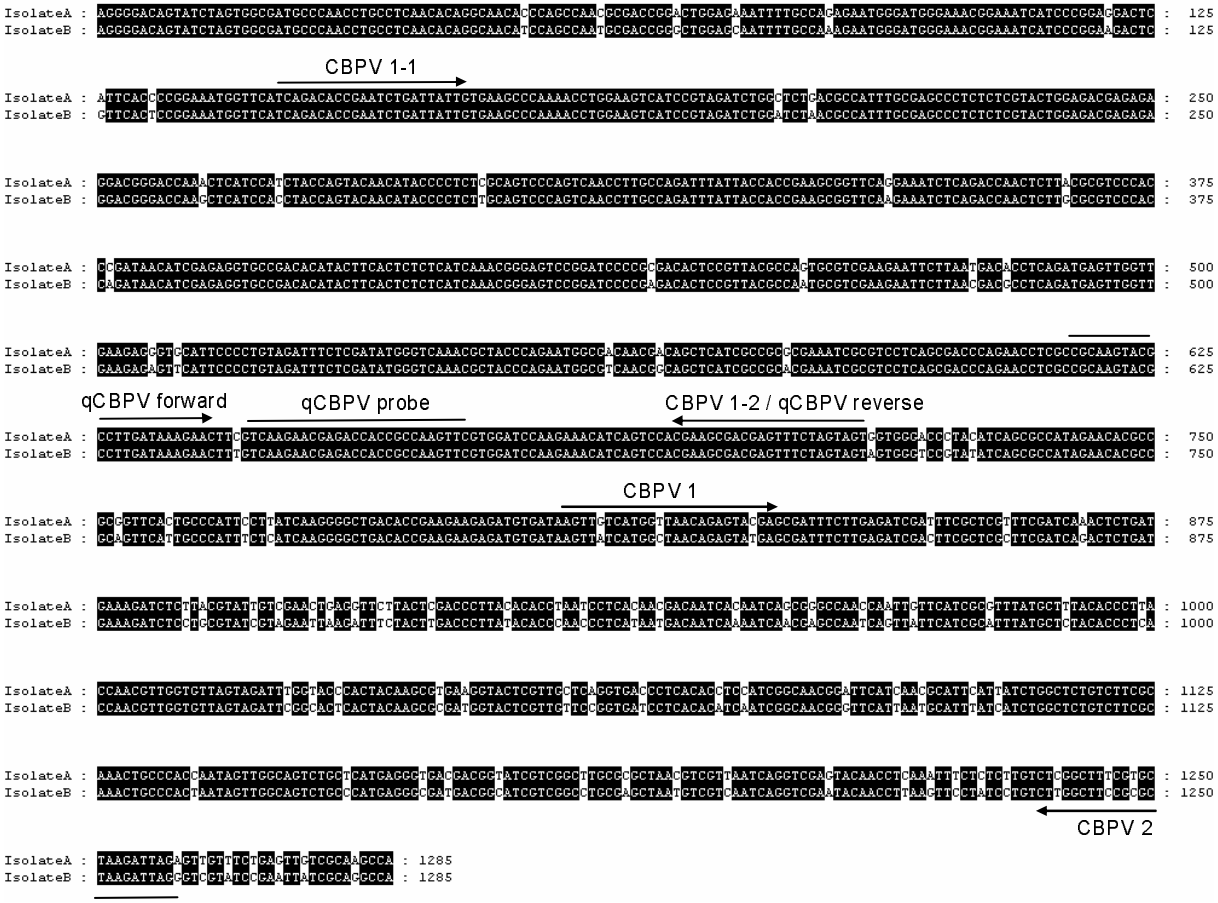
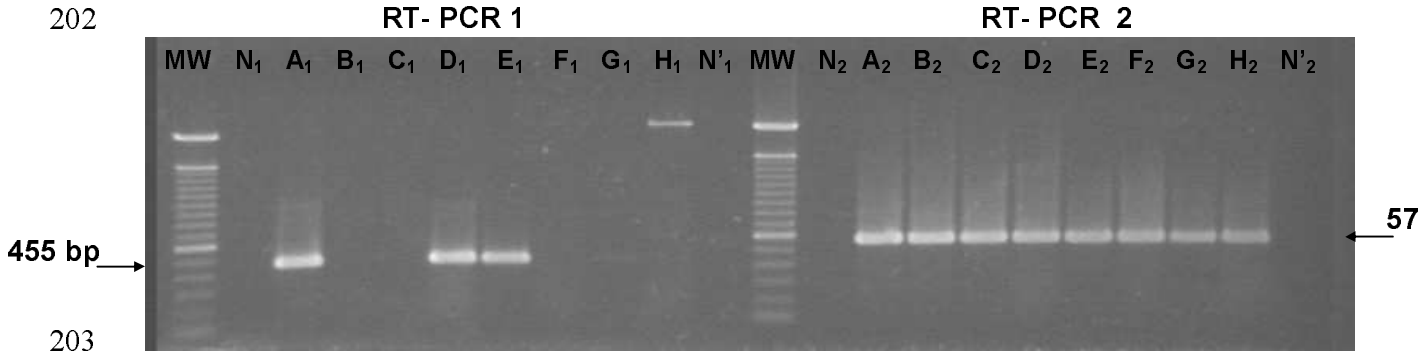

CBPV 1-1
qCBPV forward
qCBPV probe
CBPV 1-2 / qCBPV reverse
CBPV 1
CBPV 2

Figure 1

Figure 2


RT- PCR 1
RT- PCR 2
MW N1 A1 B1 C1 D1 E1 F1 G1 H1 N'1 MW N2 A2 B2 C2 D2 E2 F2 G2 H2 N'2
455 bp
57

208 Table 1 :
209

Primer name	Primer sequence	5' - position	Product size (bp)
CBPV 1	5'- AGT TGT CAT GGT TAA CAG GAT ACG AG – 3'	806	455
CBPV 2	5' - TCT AAT CTT AGC ACG AAA GCC GAG – 3'	1260	
CBPV 1-1	5' - TCA GAC ACC GAA TCT GAT TAT TG – 3'	147	570
CBPV 1-2	5' - ACT ACT AGA AAC TCG TCG CTT CG – 3'	716	

210
211 Nucleotide positions refer to the sequence of 1285 b of the CBPV reference isolate.
212