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

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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
design new RT-PCR primers able to detect all these viral samples and thereby improve
the CBPV diagnosis.

In this paper, we describe the development of a new RT-PCR diagnostic test for the
detection of CBPV isolates presenting genomic variability. The 5' RACE System
(Invitrogen) was used on the reference isolate (Isolate A) to obtain an additional
sequence from the one reported previously (Ribière et al., 2002). Then, a preliminary
study of the genomic variability of seven CBPV viral isolates was begun. Bee samples
were collected from honey bee colonies presenting typical symptoms of chronic paralysis,
located in the French departments of Aisne, Alpes-Maritimes, Drôme, Essonne and
Rhône. Sample preparation, RNA extraction and cDNA synthesis were performed as
described previously (Ribière et al., 2002; Blanchard et al., 2007). Several primers were
tested and two sets of primers were retained, allowing to obtain by assembling a
sequence of 1285 bases for each isolate. Using these 2 sets of primers, a next PCR was
performed with a high-fidelity DNA polymerase (Platinum® Pfx DNA Polymerase,
Invitrogen). The PCR products were purified using the Qiaquick PCR purification kit
(Qiagen) and sequenced (Millegen, France). The 1285 bp sequence, located in the
putative viral RNA-dependent RNA polymerase (RdRp) gene region of CBPV, was
aligned using the Clustal W method in MegAlign in the Dnastar package.

Isolate A is the CBPV reference isolate obtained by infectivity test as previously
described (Ribière et al., 2000). Isolate B is the sequence obtained from field samples
which presented the most variability with the reference isolate. Analysis of sequence pair
distances using the Clustal W method revealed 8.7% divergence between the two
sequences, mainly at the 3' region of this sequence whereas analysis of the amino acid
sequence revealed only 2.5% divergence between the two sequences. The alignment of
the sequence of the two isolates is illustrated in Figure 1.

These initial observations demonstrated the nucleotide sequence variability of the 3'
region, where primer pair CBPV 1 and CBPV 2 used for the first RT-PCR test was
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 (N1 and N2 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 B2) 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

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Captions to figures

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

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

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

RT-PCR 1  RT-PCR 2

MW  N1  A1  B1  C1  D1  E1  F1  G1  H1  N1'  MW  N2  A2  B2  C2  D2  E2  F2  G2  H2  N2'

455 bp  57
Table 1:

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

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