Evaluation of a real-time two-step RT-PCR assay for quantitation of Chronic bee paralysis virus (CBPV) genome in experimentally-infected bee tissues and in life stages of a symptomatic colony.

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EVALUATION OF A REAL-TIME TWO-STEP RT-PCR ASSAY FOR QUANTITATION OF CHRONIC BEE PARALYSIS VIRUS (CBPV) GENOME IN EXPERIMENTALLY-INFECTED BEE TISSUES AND IN LIFE STAGES OF A SYMPTOMATIC COLONY.

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Abstract

A two-step real-time RT-PCR assay, based on TaqMan technology using a fluorescent probe (FAM-TAMRA) was developed to quantify Chronic bee paralysis virus (CBPV) genome in bee samples. Standard curves obtained from a CBPV control RNA and from a plasmid containing a partial sequence of CBPV showed that this assay provided linear detection over a 7-log range (R^2>0.99) with a limit of detection of 100 copies, and reliable inter-assay and intra-assay reproducibility. Standardisation including RNA purification and cDNAs synthesis was also validated.

The CBPV TaqMan methodology was first evaluated by quantifying the CBPV genomic load in bee samples from an experimental infection obtained by topical application. Up to 1.9×10^{10} CBPV copies per segment of insect body (head, thorax and abdomen) were revealed whereas a lower CBPV genomic load was detected in dissected organs such as mandibular and hypopharyngeal glands, brain and alimentary canal (up to 7.2×10^{6} CBPV copies). The CBPV genomic loads in different categories of bees from a hive presenting the trembling symptoms typical of Chronic paralysis were then quantified. Significantly higher CBPV loads were found in guard, symptomatic and dead bees (up to 1.9×10^{13} CBPV copies) than in forager, drones and house bees (up to 3.4×10^{6} CBPV copies). The results obtained for symptomatic or dead bees support the correlation between high CBPV genomic load and pathology expression. Moreover, the high CBPV genomic load revealed in guard bees highlights the possible pivotal role played by this category of bees in CBPV infection.

Keywords: Chronic bee paralysis virus (CBPV), Real-time PCR, Quantitation, Infection dynamics, *Apis mellifera*.

1. Introduction

Chronic bee paralysis virus (CBPV) is classified as a multipartite, positive-strand RNA virus. Its family and genus are currently unassigned. CBPV was first isolated in 1963 (Bailey *et al.*, 1963) and characterized in 1968 (Bailey *et al.*, 1968). CBPV is the aetiological agent of an infectious and contagious disease of adult honey bees (*Apis mellifera* L.) known as Chronic paralysis (Ball & Bailey, 1997). Chronic paralysis is characterized by clusters of trembling, flightless, crawling bees and by individual black, hairless bees standing at the hive entrance (Bailey *et al.*, 1983). This pathology is known to induce significant losses in honey bee colonies (Bailey *et al.*, 1963; Ball & Bailey, 1997; Allen & Ball, 1996). Current diagnosis of the clinical disease is based on an AGID test (Agarose Gel ImmunoDiffusion) (Ribière *et al.*, 2000; Ball, 1999), recently complemented by RT-PCR (Ribière *et al.*, 2002), which has improved the detection sensitivity of CBPV, even in asymptomatic hives. Although these techniques are used to characterize the CBPV status of hives, they cannot estimate the level of CBPV infection. The quantitation of CBPV should permit a better understanding of the viral infection both in individual bees and in the hive.

Real-time RT-PCR detection methods have been recently developed for the detection and quantitation of bee viruses (Chen *et al.*, 2005b; Tentcheva *et al.*, 2006; Chantawannakul *et al.*, 2006). This paper describes a real-time two-step RT-PCR based on the TaqMan methodology (Heid *et al.*, 1996; Holland *et al.*, 1991; Livak *et al.*, 1995) to quantify CBPV. Standard curves from a viral RNA control and a plasmid containing a partial sequence of CBPV genome were used to obtain an absolute quantitation of CBPV. The specificity and reproducibility of the method were validated from the standard DNA curve. An experimental infection obtained by contact with purified virus was used to evaluate the method and estimate the CBPV distribution in bee tissues. The method was then applied to assess virus distribution in different categories of bees sampled from a colony with Chronic paralysis.

2. Materials and methods

2.1 Sample preparation, RNA extraction and cDNA synthesis

Adult bees were individually crushed in 1 ml of 0.01M phosphate buffer pH 6.8 in a tenbroeck grinder (Wheaton Sciences Products). The grinder was machine washed after treating with RNA and DNA decontaminant (LTK008). Hemolymph samples were collected by puncturing the intersegmental membrane at neck level, before organs dissection, using a Pasteur pipette (Fluri *et al.*, 1982) and diluted ten fold with phosphate buffer. Body segments (head, thorax, abdomen), organs dissected under binocular magnifier (mandibular and hypopharyngeal glands, brain, alimentary canal) and pooled brood samples (eggs, larvae, pupae) were washed once with phosphate buffer, then crushed in a microcentrifuge tube using a piston pellet (Eppendorf) in phosphate buffer.
The homogenate was first centrifuged at 8000g for 10 min. The supernatant was recovered, centrifuged again at 8000g for 10 min. Two hundred µl of the final supernatant were then subjected to RNA extraction using the "High Pure Viral RNA Kit" (Roche diagnosis) according to the manufacturer's recommendations. First strand cDNA was synthesized from extracted RNA as described by Ribière et al. (2002). Five µl of the cDNA were then used as template for the CBPV TaqMan PCR.

2.2 Quantitative real-time PCR protocol by TaqMan assay

The primers and probe were designed by Sigma-Proligo. The chosen primer set amplifies a 101 bp fragment and is located in the putative viral RNA polymerase gene region of CBPV (Ribière *et al.*, 2002).
The forward primer was 5'-CGCAAGTACGCCTTGATAAAGAAC and the reverse primer was 5'-ACTACTAGAAACTCGTCGCTTCG. The TaqMan probe was 5'-TCAAGAACGAGACCACCGCCAAGTTC, labelled with the fluorescent reporter dye FAM

(6-carboxyfluorescein) at the 5' end and with the fluorescent quencher dye TAMRA at the 3' end.

The Platinum® Quantitative PCR SuperMix-UDG (PQM 2X, Invitrogen) was used for the amplification reaction of the TaqMan PCR. The PCR reaction contained 1X PQM, 300 nM of each primer, 200 nM of the probe, 2 mM of $MgCl_2$ (final $MgCl_2$ concentration of 5 mM) and 5 µl of standard template (RNA control or plasmid) or cDNAs in a 25 µl total reaction volume. The reaction was performed in a 25 µl SmartCycler® Tube (Cepheid, Instrumentation Laboratory). The thermal cycling conditions were 2 min at 50°C (action of uracil-N-glycosylase (UDG) to degrade any carryover DNA amplified from previous reactions), 2 min at 95°C (activation of Platinum *Taq* DNA polymerase and degradation of UDG), followed by 40 cycles consisting of denaturing at 95°C for 10 s, annealing at 50°C for 10 s and extension at 72°C for 20 s. The SmartCycler® II System (Cepheid) was used for amplification and detection.

2.3 Preparation of viral RNA and plasmid DNA standards for calibration of the CBPV TaqMan PCR assay

A viral RNA control was obtained from the heads of sick bees, experimentally infected by inoculation as previously described by Ribière et al (2000). Based on the extracted RNA concentration determined by UV spectrometry, homogenates were then diluted to obtain 0.8×10^7 to 0.8 RNA copies per µl. Two hundred µl of each dilution were subjected to RNA extraction and cDNA synthesis. A RNA standard curve for the range of 10^8 to 10 RNA copies per reaction was generated by analysing 5 µl of each cDNA sample by TaqMan PCR.

A standard DNA curve was generated with a 3.82 kb plasmid, obtained by cloning a 800 bp PCR fragment located in the putative viral RNA polymerase gene of CBPV into the pGEM®-T Easy vector (Promega). Based on the DNA concentration determined by UV spectrometry, stock solutions were prepared from serial dilutions containing 0.2×10^8 to 2

DNA copies per µl in TE buffer. A standard DNA curve for the range of 10^8 to 10 DNA copies per reaction was generated by analysing 5 µl of each dilution by TaqMan PCR.

In both cases, the homogenate obtained from the heads of infected bees described above, was subjected to RNA extraction and cDNA synthesis and used as a positive control. A negative control (template-free) was included.

2.4 Evaluation of the method

The two standard curves (viral RNA and plasmid DNA) were compared for the efficiencies, coefficients of correlation and results obtained for the positive control.

The limit of detection of the CBPV TaqMan PCR was compared with that of the qualitative PCR test previously described by Ribière et al. (2002), by testing the viral RNA and plasmid DNA standard prepared in dilution as detailed above. The primers were CBPV1: AGTTGTCATGGTTAACAGGATACGAG and CBPV2: TCTAATCTTAGCACGAA AGCCGAG. After conventional PCR, the amplified products (455 bp) were analysed in an ethidium bromide-stained 1.5% agarose gel.

The specificity of the CBPV TaqMan PCR was assessed by testing cDNAs obtained from ABPV (Acute bee paralysis virus), SBV (Sacbrood virus), BQCV (Black queen cell virus) and DWV (Deformed wing virus) samples, checked as highly positive by serial dilution in the respective specific RT-PCR tests. The primers used in the RT-PCR tests either had been previously described for ABPV (Bakonyi *et al.*, 2002) and SBV (Grabensteiner *et al.*, 2001), or designed in our lab for BQCV (BQCV 3: GGTGCAAGTCTCTTCCTAG and BQCV 4: AATAACCTGAAAGGCCAAGAG) and DWV (DWV3: GGTCCGCGGCTAAGAT TGTA and DWV4: CGGCTGTTTGATGGAAGAAGTT).

The reproducibility of the CBPV TaqMan PCR assay was demonstrated by evaluating the intra- and inter-assay variability of the C_T values obtained after amplification of 10-fold serial dilutions of the plasmid DNA standard ranging from 10^1 to 10^8 copies per reaction. Intra-assay reproducibility was evaluated by analysing three replicates of the 10-fold serial dilutions of plasmid during the same experiment. Inter-assay reproducibility was

156 estimated by testing serial dilutions of the same standard in ten independent
157 experiments, performed on different days.
158 The efficiency of RNA purification and cDNA synthesis was monitored by testing the
159 positive control (RNA purification followed by cDNA synthesis) ten times and measuring
160 the variability of the C_T values. In addition, 10 pooled bees from a field sample were
161 crushed, divided into 10 sub-samples, and then tested ten times (RNA purification
162 followed by cDNA synthesis) to determine sample preparation efficiency. Finally, the
163 effect of crushing was evaluated by homogenizing healthy bees for half the normal
164 homogenization time, checking the negativity, loading with diluted purified virus and then
165 completing the homogenization. Ten sub-samples were tested (RNA purification followed
166 by cDNA synthesis) to assess the effect of homogenization on the virus.
167
168 2.5 Validation of the CBPV TaqMan PCR method on experimentally- and naturally-
169 infected bees
170 First, paralysed bees were obtained from an experimental model based on bee infection
171 by topical application as described by Bailey et al (1983). Briefly, bees anaesthetized with
172 carbon dioxide, were infected by contact with 3 µl of a one thousand fold diluted viral
173 purification on the shaved thorax (Group 1). The CBPV genomic load of this inoculum
174 was determined. Negative controls were obtained by replacing the inoculum with
175 physiological solution (Group 2). Trembling symptoms and mortalities were recorded daily
176 until day 12 post-infection. Hemolymph samples were collected, at day 8 post-infection,
177 from bees of both groups. The out-flowing hemolymph from 20 bees was pooled in chilled
178 tubes and diluted ten fold with phosphate buffer. Four bees from each group were killed
179 between days 8 and 9 post-contact and tissues of mandibular and hypopharyngeal
180 glands, brain and alimentary canal were dissected. At the same time, the head, thorax
181 and abdomen from 4 other bees of both groups were also collected.
182 Secondly, bees were collected from a single bee colony in our experimental apiary where
183 trembling and crawling bees were observed at the hive entrance. Samples of

184 symptomatic trembling bees were taken from the flight board. The organs from 2 of these
185 bees were dissected and hemolymph collected from 20 other bees. These samples were
186 subjected to the same analyses as the experimentally-infected bee samples.
187 Several categories of adult bees were then sampled i) on the flight board: guards (10),
188 pollen and nectar foragers (10), symptomatic (10) and dead bees (10) and ii) from inside
189 the hive: drones (5), workers on a brood frame (10), workers on a honey frame (20) and
190 emerging bees (5). Pools of pupae (5), larvae (10) and eggs (10) were also sampled.
191
192 2.6 Statistical analysis.
193 The CBPV genomic loads obtained from experimentally- and naturally-infected bees were
194 analysed by the Two sample KS (Kolmogorov-Smirnov) or the Kruskal-Wallis non-
195 parametric tests using the SYSTAT 9 computer software package (SPSS Inc.).

196 ## 3. Results

197

198 3.1 Standardisation of the CBPV TaqMan PCR assay

199 The assay was calibrated using a viral RNA control and a plasmid DNA control as
200 standards. The two standard curves generated from the amplification plot of a 10-fold
201 serial dilution (Figure 1) showed a linear correlation between the C_T values and the initial
202 RNA and DNA load over a 7-log range ($R^2 > 0.99$). The slopes of the RNA and DNA
203 standard curves were –0.294 and –0.301 respectively, indicating that the efficiency of the
204 CBPV TaqMan PCR was close to 100%, the slope of a linear regression curve being –
205 0.303 for a theoretical 100% effective PCR (SmartCycler user's manual, Instrumentation
206 Laboratory). No amplification was detected in the negative control.
207 The RNA concentration of the positive control, determined by spectrometry, was 2.6 x
208 10^{10} CBPV RNA copies per µl. The estimated CBPV genomic load, according to the RNA
209 and DNA standard curves respectively was 1.14 x 10^{10} and 2.07 x 10^{10} CBPV copies per
210 µl. These results confirm the accuracy of the both standard controls and validate the use
211 of the DNA standard curve for the CBPV TaqMan PCR. The limit of detection of the
212 CBPV TaqMan PCR was 100 CBPV genome equivalent copies, compared to
213 conventional PCR for which the limit of detection of CBPV genome from viral RNA and
214 plasmid DNA control was 10^4 copies (data not shown).

215

216 3.2 Specificity and reproducibility of the CBPV TaqMan PCR

217 CBPV specificity was confirmed by a Blast search on the amplicon (101 bp) generated by
218 the TaqMan PCR. No significant similarity was found.
219 Furthermore, no amplification was detected when this TaqMan PCR was performed on
220 cDNAs obtained from ABPV (Acute bee paralysis virus), SBV (Sacbrood virus), BQCV
221 (Black queen cell virus) or DWV (Deformed wing virus) samples.

222 The coefficient of variation (CV) of the mean C_T values obtained for the DNA standard
223 curve ranged from 0.13 to 1.39% within a run (intra-assay reproducibility) and from 1.21
224 to 2.35% from run to run (inter-assay reproducibility).
225 The estimated mean CBPV genomic load of the positive control was at 1.43×10^{10} CBPV
226 copies per µl of RNA extract, close to the concentration of 2.6×10^{10} CBPV RNA copies
227 per µl determined by UV spectrometry. The coefficient of variation of C_T evaluated from
228 10 independent experiments (RNA extraction followed by cDNA synthesis) was 0.99%.
229 The estimated mean CBPV genomic load from field sampled bees was 5.9×10^4 CBPV
230 copies per bee. The coefficient of variation of C_T evaluated from 10 independent
231 experiments (RNA extraction from homogenized sub-samples followed by cDNA
232 synthesis) was 0.82%. Furthermore, the C_T results obtained from crushed healthy bees,
233 pre-loaded with purified virus (10 replicates) showed a coefficient of variation of 2.17%.
234
235 3.3 Performance of the CBPV TaqMan PCR assay on body segments and tissues of
236 experimentally- and naturally-infected bees
237 The estimated CBPV genomic load in the inoculum applied to the bee thorax was $1.8 \times$
238 10^8 CBPV copies. Few trembling and weakening symptoms were observed 7 to 8 days
239 post-contact in the infected bees group and all bees died 8 to 9 days post-contact. No
240 symptoms occurred in the control group and the survival rate at day 12 was 72%.
241 The CBPV genome equivalent copy numbers (GECN) determined from samples from the
242 infected group are given in Figure 2. The CBPV GECN in samples of mandibular and
243 hypopharyngeal glands, brain and alimentary canal collected from 4 bees ranged from
244 3.7×10^4 to 7.2×10^6 copies with respective means of 2.5×10^5, 6.8×10^5 and 2.1×10^6
245 copies. Higher GECN, ranging from 4.4×10^{10} to 1.1×10^{12} copies, were observed in the
246 dissected organs of the two symptomatic bees sampled from the flight board of the hive.
247 The CBPV GECN in samples of abdomen, thorax and head collected from four other
248 experimentally-infected bees ranged from 1.8×10^5 to 1.9×10^{10} copies with respective
249 means of 4.1×10^8, 2.4×10^9 and 4.9×10^9 copies. The CBPV genomic load in these

250 body segment samples was up to 10^3 fold higher than in the dissected tissue samples
251 and this difference was significant (p=0.018). However, no significant difference was
252 observed within the groups.
253 The CBPV genomic loads in hemolymph samples collected from experimentally- and
254 naturally-infected bees were 2 x 10^{10} and 4.1 x 10^9 copies of CBPV per µl respectively.
255 No CBPV genomic load was detected in any sample (organs, body sections, or
256 hemolymph) from the control group.
257
258 3.4 Performance of the CBPV TaqMan PCR assay on various categories of bees
259 sampled from a symptomatic hive
260 The CBPV genomic loads obtained for each category of bees from a hive showing typical
261 symptoms of paralysis are given in Figure 3. The detected CBPV genomic load was
262 significantly higher in guard, symptomatic and dead bees than in drones, foragers, house
263 bees and brood collection (p<0.001). The mean CBPV genomic load in the first category
264 was as high as 10^{12} copies per bee, and ranged from 10^4 to 10^6 copies per bee in the
265 second category. No significant difference was observed between guard, symptomatic
266 and dead bees whereas the CBPV GECN in forager bees was significantly different
267 (p=0.012) to that of house bees sampled from brood and honey frames. The CBPV
268 genomic loads in pupae, larvae and eggs remained relatively low and did not exceed 2 x
269 10^3 CBPV copies per individual.

4. Discussion

In this study, a real-time two-step TaqMan RT-PCR assay was developed to quantify the CBPV genomic load in bee samples and determine the distribution of CBPV infection both within the bees and within the hive. A TaqMan probe with primers located in the putative viral RNA polymerase gene of CBPV was used. The standard curves generated with the viral RNA control and with the plasmid containing a partial sequence from the CBPV genome showed that quantitation of this genome was linear over 7 orders of magnitude. The efficiency of both standard curves and their good correlation was confirmed. Quantitation of the positive control from CBPV-infected bee heads gave similar results for both methods compared to the value obtained by UV spectrometry. These results validate the use of the DNA standard curve to quantify CBPV in bee samples. The limit of detection of this TaqMan PCR method was 10^2 CBPV genome equivalent copy number (GECN), representing an improvement of the conventional PCR previously developed in our laboratory (Ribière *et al.*, 2002), for which the limit of detection was 10^4 CBPV copies. The intra-assay and inter-assay reproducibility studies showed the high reproducibility of the method for the standard curve (from 0.13% to 1.39% and from 1.21% to 2.35% respectively), and for the efficiency of RNA purification and cDNA synthesis from the positive control (0.99%). The high reproducibility of the homogenisation technique was validated by the 10 replicates of field samples (0.82%) and loaded samples (2.17%).

CBPV specificity was demonstrated in relation to ABPV (Acute bee paralysis virus), SBV (Sacbrood virus), BQCV (Black queen cell virus) and DWV (Deformed wing virus) samples. These sensitivity, specificity and reproducibility results indicate that this assay can be used to quantify the CBPV genome.

Real-time PCR detection methods have been developed recently for the detection and quantitation of the main honey bee viruses in *Varroa destructor* (Chantawannakul *et al.*, 2006), or DWV in honey bees and/or *Varroa destructor* (Chen *et al.*, 2005b; Tentcheva *et*

297 *al.*, 2006). This is the first report to describe a real-time PCR detection method for the
298 detection and quantitation of CBPV in honey bees.
299 The performance of our CBPV TaqMan PCR assay was assessed on bees infected by
300 topical application of the virus. Some bees only reproduced trembling symptoms of
301 chronic paralysis during this experiment but all were dead by day 9 post-contact. An
302 infection by intra-thoracic injection was also conducted, as described by Ribiere et al.
303 (2002). In this case, all bees developed trembling symptoms from day 5 post-inoculation.
304 We chose to analyse samples obtained by contact contamination, which corresponds to
305 the supposed natural way of infection. Bailey et al. (1983) showed that CBPV could be
306 transmitted when applied experimentally to the cuticle surface, freshly denuded of its
307 hairs. They hypothesized that the Chronic paralysis virus was transmitted by direct
308 contact with the epidermal cytoplasm and that, in nature, paralysis might be transmitted
309 by contact when healthy bees are crowded with infected individuals.
310 The CBPV quantitative PCR results obtained in this model revealed high genomic loads
311 in the head, thorax and abdomen of infected bees, reaching 1.9×10^{10} CBPV copies in an
312 experimentally-infected bee head. The estimated load in the inoculum was 1.8×10^{8}
313 CBPV copies so the CBPV multiplied actively in bees following contact. This corroborates
314 previous works that suggested the existence of a high concentration of CBPV in the head
315 of infected bees (Bailey & Milne, 1969; Ribière *et al.*, 2002). Although results showed a
316 significant difference between body segments and dissected organs ($p<0.05$), no
317 significant difference was observed within the two sets of samples. Lower results were
318 found in dissected organs with up to 7.2×10^{6} CBPV copies in the alimentary canal of one
319 infected bee. However, higher GECN (up to 1.1×10^{12} copies) were revealed in the
320 dissected organs of naturally paralysed bees.
321 Hemolymph samples from experimentally- or naturally-infected bees presented high
322 genomic loads of up to 2×10^{10} and 4.1×10^{9} CBPV copies per µl respectively. We
323 confirm the previous observations of Chen et al. (2006) who described CBPV in
324 hemolymph, and demonstrate for the first time the presence of high CBPV loads in this

tissue. A high CBPV load in the hemolymph of experimentally-infected bees confirms the efficiency of CBPV infestation of bees by topical application. However, the virus loads were higher in the organs of naturally-infected bees than in experimentally-infected bees.

We then validated our CBPV TaqMan methodology by conducting a second study in a symptomatic hive in our apiary, that exhibited trembling and crawling bees at the hive entrance. Various categories of bees were analysed to evaluate the CBPV genomic loads and estimate CBPV distribution within the hive.

The results showed that the CBPV genomic load was significantly higher in guard, symptomatic and dead bees (up to 1.9×10^{13} CBPV copies) than in forager, drones and workers sampled inside the hive (up to 3.4×10^{6} CBPV copies). The results obtained for symptomatic or dead bees confirm the strong correlation between high CBPV genomic load and pathology expression. Surprisingly, the highest CBPV genomic loads were not observed in the oldest bees (the foragers) but in the guard bees, which addresses the question of their role in infection. In fact, the CBPV genomic loads differed significantly between guards and foragers ($p<0.05$). It could be hypothesized that the high CBPV genomic load detected in guards sampled from the flight board of a highly symptomatic colony might be related to the higher frequency of close contacts of guards, compared to foragers, with other bees. Furthermore, the significantly lower genomic loads observed in drones and house bees sampled inside the hive, compared to forager bees ($p<0.05$), is in accordance with the fact that the disease is essentially described in older adult bees (Ball, 1999).

The quantitative CBPV PCR results obtained in pupae, larvae and eggs are concordant with the results obtained in brood samples by Chen et al. (2006), who hypothesized the possible transmission of CBPV from the queen to her progeny (Chen *et al.*, 2005a). However, the CBPV genomic load in these various brood samples remained relatively low, and within the limit of sensitivity of our TaqMan CBPV assay. Further investigations on the queen and her progeny will be necessary to more precisely assess the vertical transmission of CBPV and its epidemiological role.

353 In conclusion, the CBPV TaqMan RT-PCR assay developed in this study provides a
354 sensitive, specific and reproducible method for the quantitation of CBPV genomic RNA.
355 Preliminary assessment of the CBPV genomic loads in bee samples shows that this
356 method can be used to monitor chronic paralysis infection in colonies. Samples of
357 experimentally- and naturally-infected bees were studied during this work. However,
358 CBPV can persist in apparently healthy colonies, without typical symptoms (Bailey *et al.*,
359 1981) and these “non-apparent infections” can be detected by molecular methods
360 (Ribière *et al.*, 2002). CBPV infection should now be studied in parallel in symptomatic
361 and asymptomatic hives to compare the CBPV viral load, and its dynamics and
362 distribution between individuals of the same colony. The question of correlation of the
363 CBPV load threshold with overt disease could then be addressed and, in a broader
364 context, the risk factors associated with the disease.

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372 beekeeping products.

Captions to figures

Figure 1.

RNA and DNA standard curves of CBPV TaqMan PCR assay using a FAM-TAMRA labelled TaqMan probe obtained with a 10-fold serial dilution (10^8-10 copies) of a viral RNA control from the heads of sick bees (experimentally infected by inoculation) and a 3.82 kb plasmid including a 800 bp fragment located in the putative viral RNA polymerase of CBPV, respectively.

The standard curves were obtained by linear regression analysis of the C_T measured for each amplification (x-*axis*) versus the Log copy number for each standard dilution (y-axis). The slopes of the two standard curves (RNA, -0.294 and DNA, -0.301) and the correlation coefficient are indicated (r-squared = 0.995 and 0.992, respectively).

Figure 2.

Evaluation of the CBPV genomic load by CBPV TaqMan PCR assay in organ and body contact samples from bees experimentally-infected by contact. The results are expressed as the mean CBPV genome equivalent copy number per organ and body segment collected from bees at 8 to 9 days post-contact. Bars represent the standard deviations.

Figure 3.

Evaluation of CBPV genomic loads by CBPV TaqMan PCR assay in various categories of bees from a symptomatic hive. The results are expressed as the mean CBPV genome equivalent copy number per bee. Bars represent the standard deviations.

395

Figure 1

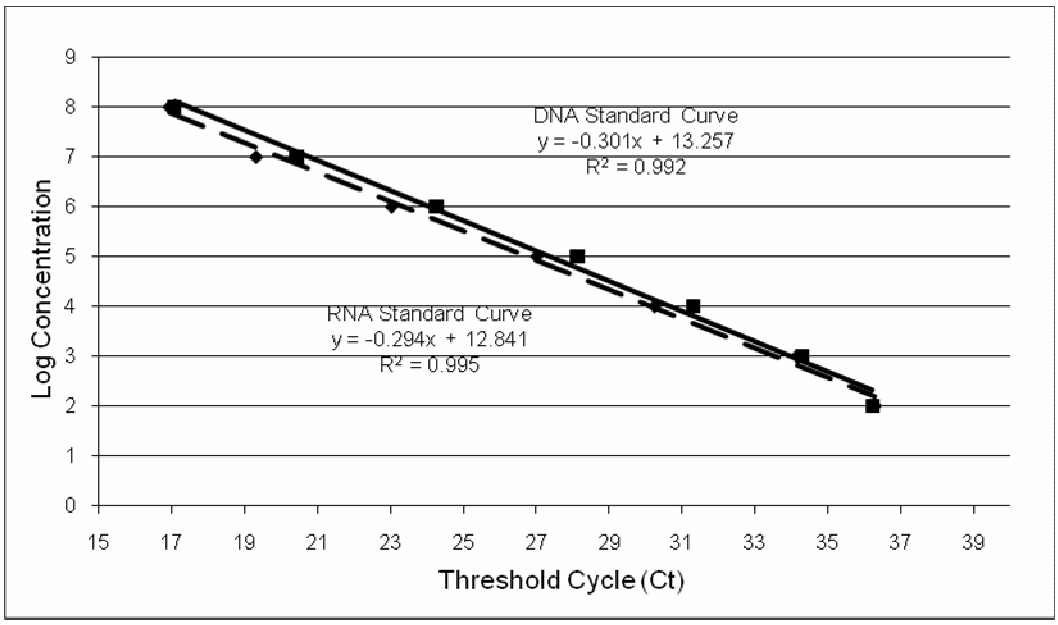

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Figure 2

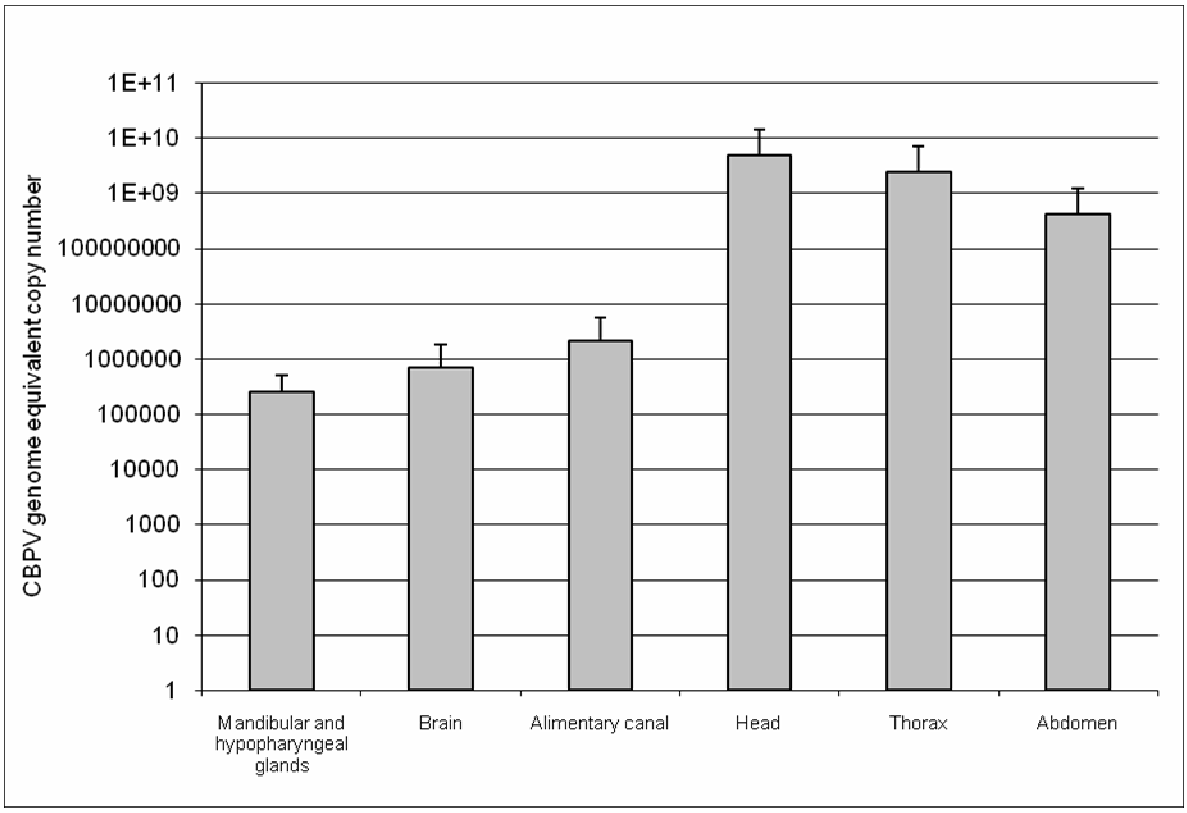
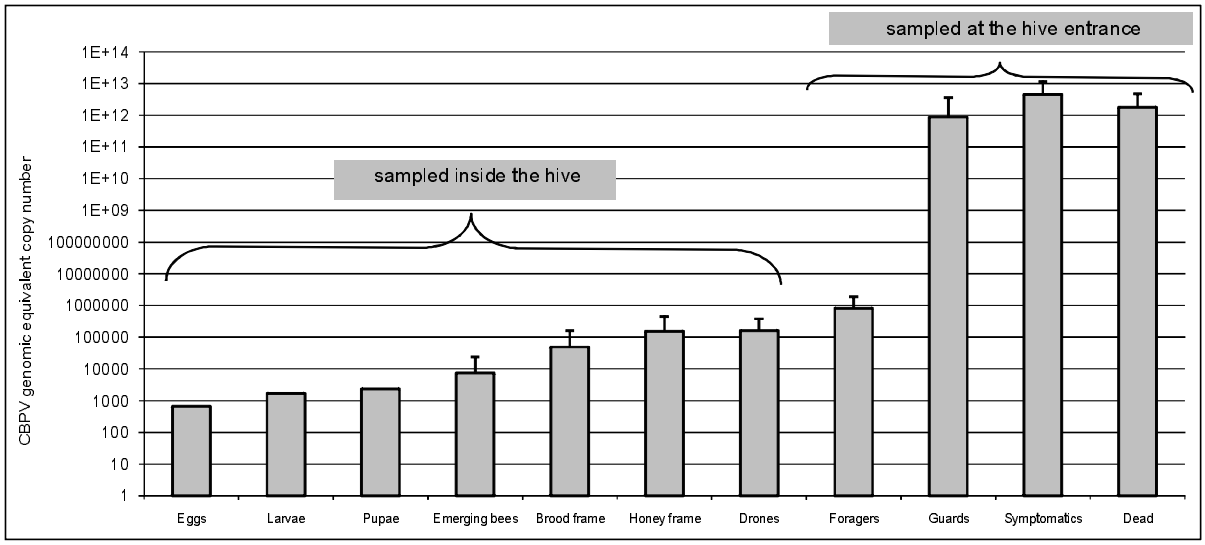


Figure 3



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