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1 **Detection of Chronic bee paralysis virus (CBPV) genome**
2 **and its replicative RNA form in various hosts and possible**
3 **ways of spread.**

4

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21

22 **Abstract**

23 Detection of Chronic bee paralysis virus (CBPV) is reported for the first time in two
24 species of ants (*Camponotus vagus* and *Formica rufa*) and in *Varroa destructor*. A
25 quantitative real-time PCR (qPCR) method was used to detect and quantify CBPV in
26 infected bees, ants and mites. A minus-strand-specific RT-PCR was used to assess viral
27 replication. These results suggest a new way by which the infection may be spread and
28 other sites of viral persistence in the close apiary environment.

29

30 Keywords: Chronic bee paralysis virus (CBPV), *Camponotus vagus*, *Formica rufa*, *Varroa*
31 *destructor*, Quantitation, minus- strand-specific RT-PCR, *Apis mellifera*.

32

33 **Short Report**

34

35 A large variety of viruses multiply in the honey bee *Apis mellifera* L. (Allen & Ball, 1996).
36 Knowledge of the spreading mechanism of honey bee pathogens within the hive and
37 apiary is essential to our understanding of bee disease dynamics. Among the viruses
38 infecting honey bees, Chronic bee paralysis virus (CBPV) is the causal agent of chronic
39 paralysis known to induce significant losses in honey bee colonies (Ball & Bailey, 1997).
40 The pathology is characterized by clusters of trembling, flightless, crawling bees and by
41 individual bees, sometimes hairless, standing at the hive entrance (Bailey et al., 1983). A
42 correlation between chronic paralysis and high viral loads of CBPV was demonstrated
43 particularly in symptomatic bees (Blanchard et al., 2007a). Moderate viral loads were also
44 demonstrated in colonies without symptoms (Blanchard et al., 2007a). To date, CBPV
45 has been detected only in *A. mellifera* (Allen & Ball, 1996) and the presence of this virus
46 has been observed on every continent (Bailey, 1967). However, several honey bee
47 viruses have been identified in the ectoparasitic mite *Varroa destructor* (Ball & Allen,
48 1988). Acute bee paralysis virus (ABPV) and deformed wing virus (DWV) have been
49 identified as major factors contributing to the mortality of bees in colonies infested by *V.*

50 *destructor* (Ball & Allen, 1988; Tentcheva *et al.*, 2004; Yue & Genersch, 2005). In addition,
51 DWV replicative RNA was detected in *V. destructor* (Yue & Genersch, 2005). Although
52 the association between mites and CBPV was not demonstrated (Ball & Allen, 1988,
53 Tentcheva *et al.*, 2004), the presence of mites in CBPV symptomatic hives was observed,
54 suggesting its potential role in the viral dissemination. Like the mite *V. destructor*, which is
55 recognised as the main vector of some honeybee viruses, other organisms could also
56 play this vector role in the honeybee. In fact, during our field observations, we noted the
57 presence of ants close to apiaries, carrying dead bees around hives. Two species of ants
58 were observed: *Camponotus vagus*, a very common ant with a world-wide distribution
59 and *Formica rufa*, present in many coniferous and mixed-coniferous forests in the
60 temperate zones of northern Europe and Asia (Jurgensen *et al.*, 2005). Both ant species
61 seek honeydew but are also carnivorous. These field observations led to the idea that
62 these other hymenoptera might disseminate the virus in the apiary environment. Our aim
63 was first to evaluate the presence of CBPV in other hosts such as mites and ants,
64 secondly to determine whether the CBPV was able to replicate in these hosts, and thirdly
65 to evaluate the genome variability of a partial sequence of CBPV between them.

66

67 **Sample collection and preparation, RNA extraction and cDNA synthesis.**

68 In this study, the honey bees, ants and mites were collected from three apiaries where
69 more or less pronounced symptoms of trembling and crawling bees were observed at the
70 entrance of the hives. Apiary 1, the laboratory's apiary, is located in the French
71 department of Alpes-Maritimes. Apiaries 2 and 3 belonged to a professional beekeeper
72 and are located 500 kilometres from apiary 1, in a forest area in the French department of
73 Haute-Loire. Sample collection and preparation are detailed in Table 1. Mite and ant
74 samples were washed 3 times by vortexing in phosphate buffer, in order to remove
75 external contamination before crushing. Samples (pool of individuals) were then crushed
76 in 0.01 M phosphate buffer pH 6.8 (homogenisation volumes are indicated in Table 1),
77 total RNA and first strand cDNA were synthesized as described by Ribière *et al.* (2002).

78 Five microliters of the cDNA were then used as template for the CBPV TaqMan PCR, or
79 the minus-strand RNA detection.

80

81 **Upgrading the CBPV real-time two-step RT-PCR assay.**

82 A real-time two-step TaqMan RT-PCR assay has recently been developed to quantify the
83 CBPV genomic load in bee samples (Blanchard et al., 2007a). This assay was adapted to
84 the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems), according to the
85 manufacturer's recommendations, to permit the analysis of a more significant number of
86 samples. The reaction was performed in duplicate using a MicroAmp Optical 96-well
87 Reaction Plate and the same set of primers and probe as previously published. The PCR
88 reaction contained 1X TaqMan Universal PCR Master Mix (UMM 2X, Applied Biosystems), 300nM of
89 each primer, 200nM of the probe and 5µl of standard template or cDNAs in a total
90 reaction volume of 25µl. The thermal cycling conditions were 2 min at 50°C (action of
91 uracil-*N*-glycosylase (UNG) to degrade any carryover DNA amplified from previous
92 reactions), 10 min at 95°C (activation of AmpliTaq Gold DNA Polymerase and
93 degradation of UNG), followed by 40 cycles consisting of denaturing at 95°C for 15s and
94 annealing/extension at 60°C for 1 min. The method was calibrated and evaluated using
95 the ABI 7500 system in the same way as with the SmartCycler II System (Blanchard et
96 al., 2007a). The two standard curves, generated as previously described from the
97 amplification plot of the 10-fold serial dilution (viral RNA and plasmid DNA) (Blanchard et
98 al., 2007a), showed a linear correlation between the C_T values and the initial RNA and
99 DNA load over an 8-log range ($R^2 > 0.99$). The slopes were -3.433 and -3.344
100 respectively and the limit of detection was 10 copies, thus allowing improvement of the
101 method. The intra-assay and inter-assay reproducibilities were checked to confirm the
102 adaptation of this method to the ABI 7500 system.

103

104 **Detection and quantitation of CBPV in various samples**

105 The CBPV genomic loads obtained for each sample from the three apiaries are given in
106 Fig. 1. The CBPV genome equivalent copy numbers (GECNs) determined from bees in
107 the three apiaries ranged from 1.3×10^6 to 8.9×10^{11} copies per bee. The external bees
108 from apiary 2, which presented trembling symptoms, revealed the higher CBPV viral load,
109 in concordance with previous results (Blanchard et al., 2007a).

110 Unexpectedly, a CBPV genomic load was detected in the mite sample from apiary 1 but
111 was relatively low with 1.4×10^4 copies per individual. No CBPV has previously been
112 detected in mites (Tentcheva et al., 2004). Moreover, significant CBPV genomic loads were
113 revealed in two species of ants. The CBPV GECNs in the three samples of *F. rufa*, found
114 in the forest apiaries 2 and 3, ranged from 2.3×10^3 to 1.8×10^7 copies per individual. The
115 CBPV genomic loads obtained for russet ant (*C. vagus*) collected from apiaries 1 and 2
116 ranged from 1.1×10^8 to 1.3×10^{11} per ant. The CBPV genomic loads attained 1.3×10^6
117 CBPV copies per pupa of *C. vagus*, whereas the CBPV genomic load of the dead alate (♀)
118 was relatively low and did not exceed 3×10^3 CBPV copies per individual. Here, for the first
119 time we report the presence of CBPV in a sample of mites and in ant samples. These
120 observations led us to estimate the viral replication in these samples by detection of the
121 minus-strand CBPV RNA.

122

123 **Detection of the replicative form of CBPV RNA (minus-strand RNA)**

124 Given that CBPV is a positive-strand RNA virus, the synthesis of minus-strand CBPV
125 RNA is carried out during viral replication. Hence, detection of the minus-strand RNA is
126 indicative of virus replication (Craggs et al., 2001; Yue & Genersch, 2005). A specific RT-
127 PCR was developed to assess the presence or absence of minus-strand CBPV RNA in
128 different samples. First strand cDNA was synthesized from the extracted RNA described
129 above, using a minus-strand specific primer. This primer consisted of a tag unrelated to
130 CBPV (lower-case) coupled to a specific primer of CBPV (upper-case) (RT ms CBPV =
131 5'-atcggaatcgccctagcttGCTTGATCTCCTCCTGCTTG-3'). The reverse transcription was
132 performed as described by Ribière et al. (2002). Furthermore, the priming specificity

133 during reverse transcription was confirmed as described by Craggs et al. (2001), using
134 rRth DNA Polymerase (Applera) at 70°C. A minus-strand-specific PCR was then
135 performed using a forward primer corresponding to the tag sequence (Tag1 = 5'-
136 ATCGGAATCGCCTAGCTTGCT-3') with another CBPV-specific oligonucleotide as the
137 reverse primer (R23 = 5'-CCCAATGTCCAAGATGGAGT-3'), which revealed a PCR
138 product size of 349 bp. The specificity of this PCR was checked by analysing the cDNAs
139 obtained with random primers and gave a negative result. At the same time, a control
140 CBPV-specific PCR, using primers R20 (5'-GCTTGATCTCCTCCTGCTTG-3') and R23,
141 was performed on these random cDNAs and gave a positive result (PCR product size of
142 331 bp). Furthermore, a semi-nested PCR was performed to confirm the presence or
143 absence of minus-strand CBPV RNA.

144 Analysis of ant (*C. vagus*), honeybee and mite (*V. destructor*) samples led to the
145 detection of RNA minus-strand related to viral replication (Fig. 2, lanes 3, 7 and 8), as in
146 ant pupae subjected to semi-nested PCR (data not shown). No minus-strand RNA was
147 detected in the *F. rufa* workers.

148

149 **Sequence analysis**

150 The PCR products obtained from the different samples (bees, ants, varroas) were
151 sequenced as described by Blanchard et al. (2007b), to check CBPV specificity and
152 compare sequences between each host. PCR primers were chosen in order to include
153 the area where the minus-strand specific primer hybridised. Alignments, using the Clustal
154 W method, revealed an intra-homology close to 100% between the different hosts of each
155 apiary, and an inter-homology of 96 to 98% between apiaries.

156

157 **Discussion**

158 These data show for the first time the presence of CBPV in hosts other than *A. mellifera*.
159 In this study we demonstrated the presence of CBPV in two species of carnivore ants (*C.*
160 *vagus* and *F. rufa*) and in *V. destructor*. Replication was demonstrated by detection of the

161 minus-strand RNA in *C. vagus* and *V. destructor* (Fig. 2, lane 5 and 8). Furthermore,
162 comparison of a partial sequence between each host (honeybee, ant, mite) revealed a
163 homology close to 100%. These results suggest that ants may help to spread the virus by
164 increasing the amount of virus inside the nest and within the apiary and may also serve
165 as a virus reservoir in the apiary environment. This would partially explain the recurrence
166 of chronic paralysis disease, which has often been observed during the summer period,
167 every year in the same apiary (Giauffret et al., 1970). The ants and varroas might
168 represent a natural reservoir for this bee-virus. There are many examples of other virus-
169 host-systems in nature. One example is that of blood-eating arthropods, referred to as
170 arboviruses (arthropod-borne virus) such as mosquitoes and ticks which can transmit
171 virus to vertebrates (Portefield, 1986, Mertens et al., 2005). Another example of a vector
172 is the aphid, which transmits more than half of all known plant viruses (Astier et al. 2001).
173 However, virus transmission between ants and honeybees has not yet been
174 demonstrated. Two hypotheses can be suggested: i) the ants may contract the virus by
175 eating infected bees; ii) the ants and bees may infect themselves while collecting the
176 *Cinara pectinatae* honeydew. Ten honeydew-specific aphid *Cinara pectinatae* were
177 therefore collected in the coniferous-forest area (apiary 1), pooled and analysed, but the
178 CBPV was not identified in this aphid (data not shown). These results raise the question
179 to know if CBPV is only a honeybee virus able to infect ants or a virus with various
180 hosts. Complementary investigations will be required to evaluate these hypotheses.
181 This is the first discovery of a bee virus able to infect ant species (*C.vagus* and *F. rufa*).
182 To our knowledge, few data have been obtained on ant viruses. Valles et al. (2004) and
183 Hashimoto et al. (2007) showed that a positive stranded RNA virus, *Solenopsis invicta*
184 *virus-1* (SINV-1), attacked all developmental stages of red fire ant (*Solenopsis invicta*).
185 Recently, Valles et al. (2007) reported the discovery of a new virus from the red fire ant,
186 provisionally named *Solenopsis invicta virus-2* (SINV-2). Whereas SINV-1 is a picorna-
187 like virus (Valles et al., 2004), phylogenetic analysis of CBPV (Olivier et al. 2007) and
188 SINV-2 (Valles et al., 2007) has not permitted their inclusion in any virus family. In our

189 case, no perceptible symptoms of trembling ants were observed in infested nests in the
190 field, although dead ants were found along with dead bees around the apiary. Analogies
191 can be drawn with the virus-transmitting mites, such as DWV, which show no disease
192 symptoms (Ball & Allen, 1988), even when replicative form RNA was revealed (Yue &
193 Genersch, 2005). Valles et al., (2007) have also reported difficulties in observing any
194 discernable symptoms of red fire ant colonies infected by SINV-2 in the field.

195 The mite *Varroa destructor* is a highly effective vector of DWV and ABPV transmission
196 among bees (Ball & Allen, 1988). This first detection of CBPV and its capacity for
197 replication in mites suggests that *V. destructor* may also possibly spread CBPV infection
198 within the hive. This is consistent with previous findings i.e. the high GECN CBPV copies
199 in bee hemolymph, the main food source of varroa (Blanchard et al., 2007a; Chen et al.,
200 2005).

201 However, further investigations are necessary in both cases to confirm the precise role of
202 ants and varroa in the spread of infection and as virus reservoir. Knowledge of how
203 CBPV spreads is fundamental to the design of an appropriate disease control program.

204

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211 d'Orientation et de Garantie Agricole" (FEOGA), in accordance with the French
212 programme for the improvement of the production and commercialisation of beekeeping
213 products.

214

215 **Captions to figures**

216

217 Table 1

218 Collection, localisation and preparation of samples from the three apiaries.

219

220 Figure 1.

221 Quantification of CBPV genomic loads by TaqMan PCR assay in various categories of
222 samples (bees, ants and varroa) from three apiaries. The results are expressed as the
223 CBPV genome equivalent copy number per individual.

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226 Figure 2.

227 Detection of CBPV in various samples by CBPV specific minus-strand viral RNA RT-
228 PCR.

229 Lane 1: PCR negative control

230 Lane 2: RT negative control

231 Lane 3: *C.vagus*

232 Lane 4: alate (♀) *C. vagus*

233 Lane 5: *F. rufa*

234 Lane 6: Pupae *C. vagus*

235 Lane 7: *A. mellifera*

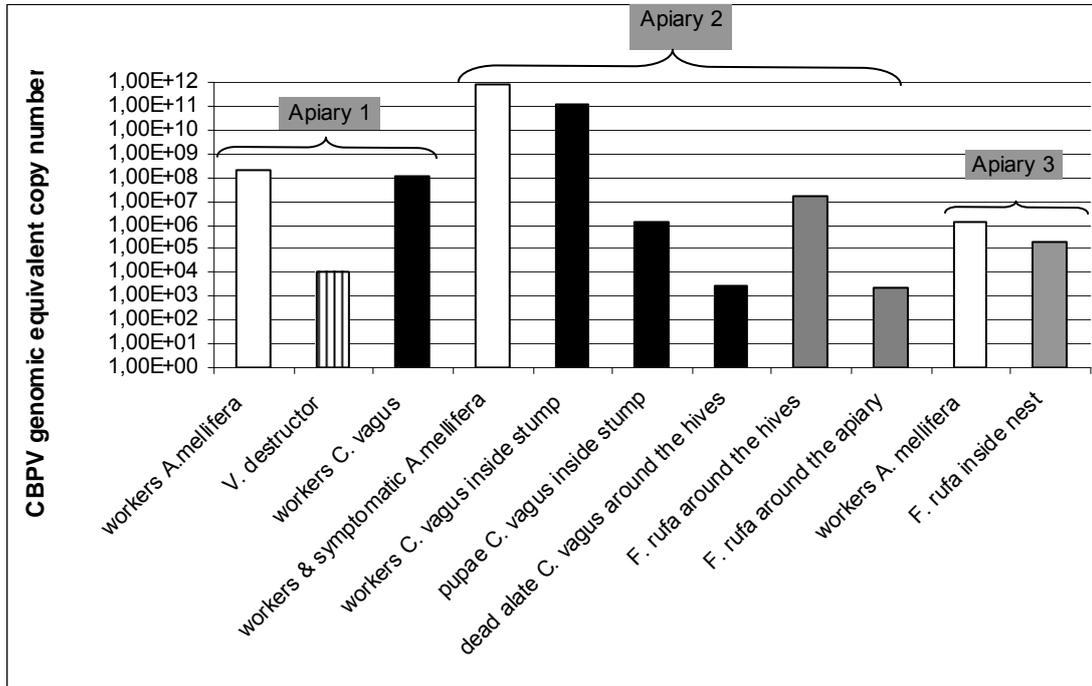
236 Lane 8: *V. destructor*

237 Lane MW: 100 bp DNA Ladder (Invitrogen)

238

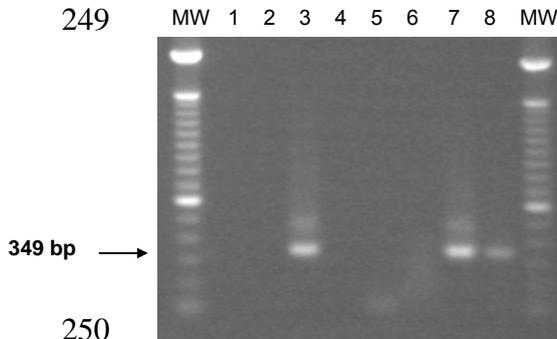
240 Table 1: Collection, localisation and preparation of samples from the three apiaries.

	Chronic Paralysis Symptoms	Sample collection (homogenisation volume)	Localisation
Apiary 1	Few	5 external bees (5ml) 19 Mites (1ml) 2 <i>C. vagus</i> workers (1ml)	on the flight board of the hive from the base of the hive during treatment with an acaricide (Amitraz) between the dead bees around the hive
Apiary 2	Yes	5 external bees (5ml) 10 <i>C. vagus</i> workers (2ml) 10 <i>C. vagus</i> pupae (1ml) 1 <i>C. vagus</i> alate ♀ (0,5ml) 10 <i>F. rufa</i> (1ml) 10 <i>F. rufa</i> (1ml)	on the flight board of the hive inside a stump (nest) 10 metres from the hives inside a stump (nest) 10 metres from the hives inside a stump (nest) 10 metres from the hives from 5 metres around the hives from 10 metres around the apiary
Apiary 3	No	5 external bees (5ml) 10 <i>F. rufa</i> (1ml)	on the flight board of the hive inside a nest 10 metres from the hives



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Figure 1 : Quantification of CBPV genomic loads by TaqMan PCR assay in various categories of samples (bees, ants and varroa) from three apiaries. The results are expressed as the CBPV genome equivalent copy number per individual.



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Figure 2: Detection of CBPV in various samples by CBPV specific minus-strand viral RNA RT-PCR.

- Lane 1: PCR negative control
- Lane 2: RT negative control
- Lane 3: *C.vagus*
- Lane 4: alate (♀) *C. vagus*
- Lane 5: *F. rufa*
- Lane 6: Pupae *C. vagus*
- Lane 7: *A. mellifera*
- Lane 8: *V. destructor*
- Lane MW: 100 bp DNA Ladder (Invitrogen)

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