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

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Abstract

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

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

Short Report

A large variety of viruses multiply in the honey bee *Apis mellifera* L. (Allen & Ball, 1996). Knowledge of the spreading mechanism of honey bee pathogens within the hive and apiary is essential to our understanding of bee disease dynamics. Among the viruses infecting honey bees, Chronic bee paralysis virus (CBPV) is the causal agent of chronic paralysis known to induce significant losses in honey bee colonies (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). A correlation between chronic paralysis and high viral loads of CBPV was demonstrated particularly in symptomatic bees (Blanchard et al., 2007a). Moderate viral loads were also demonstrated in colonies without symptoms (Blanchard et al., 2007a). To date, CBPV has been detected only in *A. mellifera* (Allen & Ball, 1996) and the presence of this virus has been observed on every continent (Bailey, 1967). However, several honey bee viruses have been identified in the ectoparasitic mite *Varroa destructor* (Ball & Allen, 1988). Acute bee paralysis virus (ABPV) and deformed wing virus (DWV) have been identified as major factors contributing to the mortality of bees in colonies infested by *V.*

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

Sample collection and preparation, RNA extraction and cDNA synthesis.

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

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

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

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

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 x 10^6 to 8.9 x 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 x 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 x 10^3 to 1.8 x 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 x 10^8 to 1.3 x 10^{11} per ant. The CBPV genomic loads attained 1.3 x 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 x 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'-atcggaatcgcctagcttGCTTGATCTCCTCCTGCTTG-3'). The reverse transcription was
132 performed as described by Ribière et al. (2002). Furthermore, the priming specificity

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

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

Sequence analysis

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

Discussion

These data show for the first time the presence of CBPV in hosts other than *A. mellifera*. In this study we demonstrated the presence of CBPV in two species of carnivore ants (*C. 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 known 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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212 programme for the improvement of the production and commercialisation of beekeeping
213 products.
214

Captions to figures

Table 1

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

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.

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)

239

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)	on the flight board of the hive
		19 Mites (1ml)	from the base of the hive during treatment with an acaricide (Amitraz)
		2 *C. vagus* workers (1ml)	between the dead bees around the hive
Apairy 2	Yes	5 external bees (5ml)	on the flight board of the hive
		10 *C. vagus* workers (2ml)	inside a stump (nest) 10 metres from the hives
		10 *C. vagus* pupae (1ml)	inside a stump (nest) 10 metres from the hives
		1 *C. vagus* alate ♀ (0,5ml)	inside a stump (nest) 10 metres from the hives
		10 *F. rufa* (1ml)	from 5 metres around the hives
		10 *F. rufa* (1ml)	from 10 metres around the apiary
Apiary 3	No	5 external bees (5ml)	on the flight board of the hive
		10 *F. rufa* (1ml)	inside a nest 10 metres from the hives

241

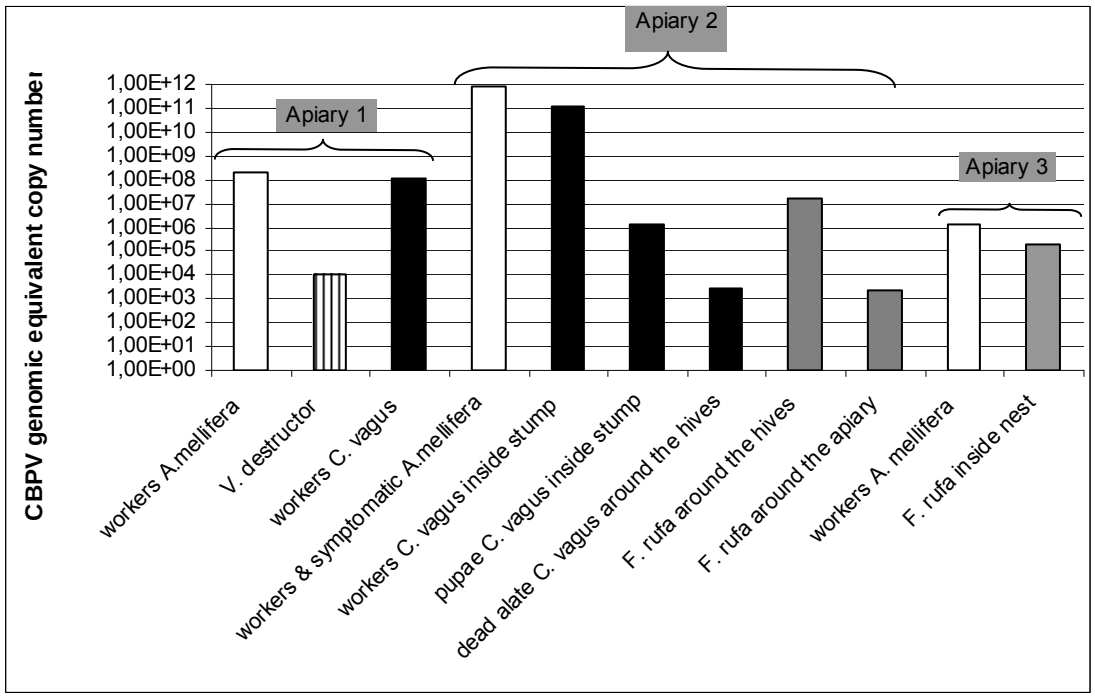

Figure 1 : Quantification of CBPV genomic loads by TaqMan PCR assay in various categories of samples (bees, ants and varroa) from three apiaries. The results are expressed as the CBPV genome equivalent copy number per individual.

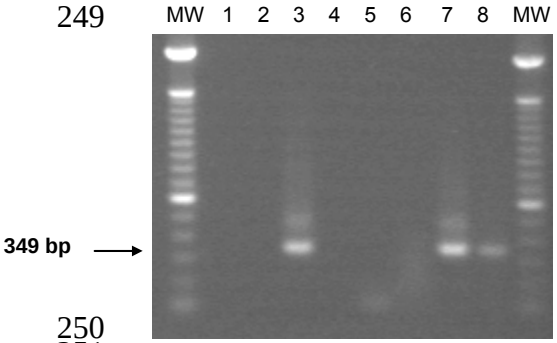

Figure 2: Detection of CBPV in various samples by CBPV specific minus-strand viral RNA RT-PCR.

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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