Apidologie 33 (2002) 339–351

DOI: 10.1051/apido:2002020

Original article

Molecular diagnosis of chronic bee paralysis virus infection

Magali RIBIÈRE*, Carole TRIBOULOT, Laetitia MATHIEU, Clément AURIÈRES, Jean-Paul FAUCON, Michel PÉPIN

AFSSA Sophia Antipolis, Unité Abeille, BP 111, 06902 Sophia Antipolis, France

(Received 29 May 2001; revised 24 January 2002; accepted 12 March 2002)

Abstract – A new RT-PCR test was developed for the diagnosis of chronic bee paralysis virus (CBPV) infection. Used in parallel with an experimental infection test, the RT-PCR test was less fastidious and allowed the detection of latent CBPV infection in colonies. The new test is based on the fact that clinical CBPV infections (but not latent infections) yield a high viral antigen load that can be easily revealed using the agarose gel immunodiffusion (AGID) test. The combination of the AGID and the RT-PCR tests allowed us to characterise the CBPV status of hives from various apiaries in France as non infected, latently infected or clinically infected. The RT-PCR test proved highly sensitive for detecting inapparent infections. It may be a useful tool for studying the epidemiology of the disease.

chronic bee paralysis virus (CBPV) / latent infection / RT-PCR diagnosis / characterisation of CBPV status / *Apis mellifera*

1. INTRODUCTION

A large variety of viruses multiply in the honey bee *Apis mellifera* L. (Ball and Bailey, 1991; Allen and Ball, 1996). One of the first to be isolated, chronic bee paralysis virus (CBPV), causes an infectious and contagious disease in two forms (Bailey, 1968). A classical form described very early in France as « maladie noire », is characterised with clusters of flightless trembling and crawling bees, and some black individuals standing at the entrance of the hive (Faucon, 1992; Faucon, 1996). This form, called Type 2 syndrome by Ball and Bailey (1997), occurs in France in spring and early summer (Giauffret et al., 1967). The other form, called Type 1 syndrome, was first described in the UK and is characterised by trembling and crawling bees and less frequent or no black individuals. This form, recently recognised in

* Correspondence and reprints
E-mail: m.ribiere@sophia.afssa.fr

France, occurs at any season (Ball and Bailey, 1997).

The CBPV may multiply to high levels in honey bees and cause significant losses (Allen and Ball, 1996). Nutritional deficiency, severe winters or bad weather conditions in summer may favour outbreaks (Bailey et al., 1983; Allen and Ball, 1996). As trembling and crawling bees can also be observed with other honey bee diseases (Sanchis and Giauffret, 1972; Mouches et al., 1984; Shimanuki, 1997; Albisetti, 1998) or follow acute or chronic intoxications with pesticides or toxic pollens (Giauffret et al., 1967; Giauffret and Lambert, 1972; Shimanuki, 1997), a laboratory diagnosis is required to determine the etiology of such syndromes.

When symptoms are observed, the presence of the CBPV antigen may easily be demonstrated using an AGID test (Agarose Gel Immunodiffusion) for the diagnosis of the clinical disease (Ribière et al., 2000). However, like many bee viruses, CBPV persists in the population as latent or inapparent infections (Ball, 1996), and AGID is not sensitive enough to reveal these latent infections. The only diagnosis method previously available was to inject extracts of apparently healthy bees into adult bees from the same colony, which may induce the disease associated with clinical signs and mortalities (Bailey et al., 1963; Bailey et al., 1981). Such infectivity tests are fastidious and time-consuming. Moreover, the lack of a honey bee cell line does not allow virus isolation. For these reasons, we developed a molecular method based on viral RNA detection as a rapid and sensitive diagnosis of latent CBPV infections.

The present study describes this test which includes RNA extraction, reverse transcription using random primers and PCR amplification. We provide a comparison of diagnosis results using this new test and the infectivity test in parallel. A combination of the RT-PCR test with the AGID diagnosis was used to monitor 33 colonies of our experimental apiary and to carry out two separate field observations during the summer of 2000.

2. MATERIALS AND METHODS

2.1. Infectivity test

The infectivity test of chronic bee paralysis was performed as previously described (Ribière et al., 2000). Briefly, 900 honey bees (*Apis mellifera* L.) were sampled in colonies without symptoms. Heads of 10 bees were crushed in a mortar at 4 °C in 5 ml physiological saline water. After centrifugation at 3 000 *g* for 30 min, 10 fold serial dilutions of the supernatant were inoculated to bees from the same colony by intra-thoracic injection (Bailey et al., 1963; Bailey, 1965). Non injected bees and bees injected with physiological saline were kept as controls (Anderson and Gibbs, 1988). All bees were kept at 30 °C in small cages supplied with sugar syrup. For each dilution, inoculation was performed in 3 cages of 30 bees. Symptoms and mortalities were closely monitored. Following chronic paralysis experimental infection, visible symptoms occurred at day 5 post-inoculation and all inoculated bees died at day 7 post-inoculation (Bailey et al., 1963; Bailey and Woods, 1977; Ball, 1996). When no symptom or mortality was observed in the inoculated bees, their colony of origin was considered to be uninfected. Honey bees from non-infected colonies were subsequently used for infectivity test applied on other colonies with symptoms (Bailey et al., 1963) and for amplifying the virus following the injection of crude or purified virus suspension as previously described (Ribière et al., 2000).

2.2. Preparation of bee samples for RT-PCR and AGID tests

Ten bee heads were crushed in a mortar at 4 °C in 0.5 ml of 0.2M potassium

phosphate buffer, pH 7.5. Half of these crude preparations were used directly in AGID tests as previously described (Ribière et al., 2000). The other part was cleared at 12 000 *g* for 20 minutes and 200 µl of the supernatant was subjected to RNA extraction in the purification kit "High Pure Viral RNA Kit" (Roche, Meylan, France). Positive control samples were realised with experimentally-infected bee extracts, and negative controls with extraction buffer only. After extraction, RNA purification was assessed by spectrophotometry at 260 nm and 280 nm.

2.3. RT-PCR test

Reverse transcription of RNAs was performed using M-MLV reverse transcriptase (Clontech, Ozyme, France). First strand cDNA synthesis was performed for 1 h at 42 ºC in reverse transcriptase buffer (50 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.5 mM dNTP's, 20 pmol of random hexamer primers, 200 U of M-MLV reverse transcriptase and 10 µl of extracted RNA, in a total volume of 20 µl. Amplification by PCR was performed using two primers designed from the sequence of the putative viral RNA polymerase gene of the CBPV (unpublished data, GenBank reference [AF375659] http://www.ncbi.nlm.nih.gov/Genbank/index.html). The primers used, CBPV1: AGTTGTCATGGTTAACAGGA TACGAG and CBPV2: TCTAATCTTAGC ACGAAAGCCGAG, amplify a 455 bp product. PCR was carried out in buffer (Platinum, Life technologies, Gibco BRL®, Cergy Pontoise, France): 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 µM of each primer, 2.5 units of Platinum® *Taq* DNA Polymerase (Life technologies, GIBCO BRL®), and 5 µl of cDNA in a total volume reaction of 50 µl. The substrate cDNA was denatured for 2 min at 95 ºC and thereafter 30 amplification cycles were carried out using the following programme: denaturation at 95 ºC for 1 min, annealing at 55 ºC for 30 s, extension at 72 ºC for 1 min, and 5 min at 72 ºC after the last cycle. Following amplification, 8 µl of PCR products were loaded with 2 µl of loading buffer (0.25% bromophenol blue, 30% glycerol), in 1.5% agarose gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.3). Gel separation results were analysed with the program computer "Bio profil" (Vilber Lourmat, Marne La Vallée, France).

We assessed the specificity of the described RT-PCR by several methods. CBPV primers were compared to the reference honey bee virus sequences available in the GenBank database using the ALIGN program (FASTA program version 2.0, W.R. Pearson & University of Virginia, USA). The first seven PCR products obtained and two PCR products from hive without clinical symptoms were sequenced (Qiagen, Hilden, Germany). The nucleotide and deduced amino acid sequences were submitted to the GenBank database under accession numbers [AF461053] to [AF461061] (http: //www.ncbi.nlm.nih.gov/Genbank/index.html). They were aligned and compared to the reference CBPV strain [AF375659] and to the honey bee virus sequences available in the GenBank database using the ALIGN program (Myers and Miller, 1988; Huang and Miller, 1991) and verified by visual inspection. In the absence of clinical symptoms, detection was validated in ten field samples by Southern blotting (Sambrook et al., 1989). PCR product of 455 pb from our reference CBPV strain was used as probe at the concentration of 10 ng/ml in hybridization buffer. Hybridization was carried out at 55 ºC overnight in an hybridization oven (Stuart Scientific, Surrey, UK). Labelling and detection were performed with the "AlkPhos direct labelling and detection" and the "CDP star detection reagent" (gene image™, Amersham pharmacia biotech, Slough, UK). To determine its sensitivity, the RT-PCR test

was applied to 10-fold serial dilutions of several samples of 10 experimentally-infected bees. This experiment was repeated two other times on new bee extracts.

2.4. Comparison of diagnostic tests

Using clinical observation and experimental infection we selected two asymptomatic and one symptomatic hives. Performance characteristics of the three tests (experimental infection, AGID and RT-PCR) were compared in samples before and after experimental infection. Characteristic samples were selected for AGID and RT-PCR tests 13 days after experimental inoculation.

2.5. Observations in an experimental apiary

Two samples of apparently healthy bees were taken from inside each of thirty three colonies (66 total) from our laboratory apiary on 11 July and 25 October 2000. They were immediately frozen at –20 °C before analysis by RT-PCR and AGID tests.

During July and August, colonies were sampled when abnormal mortality and signs evoking chronic paralysis occurred. Dead bees and bees trembling at the entrance of the hive were sampled separately. During this period, bees standing in front of the hives were sampled at least once in all colonies. These samples were analysed by AGID for CBPV diagnosis only. After these two months, colony activity was less intense and the last sample was taken on 25 October to assess the status of the hives before winter.

Colonies were treated for *Varroa destructor* using Amitraz strips (Apivar®, Biové, France) in April, and surveyed monthly for this parasite until the end of October. Each brood frame was visually inspected to detect *Varroa destructor* on bees and cells, and bees with associated symptoms such as deformed wings (DeJong et al., 1982; BowenWalker et al., 1999). Screening for nosemosis, amœba and acariosis diseases was also conducted on the 66 samples of apparently healthy bees according to previously described diagnostic methods (O.I.E, 1996). For nosemosis and amoeba diseases diagnosis, 20 bee abdomens from each colony were homogenised in 2 ml of distilled water and the preparation was examined microscopically at ×400 magnification (Steche and Ruijter, 1996). For diagnosis of *Acarapis woodi*, 20 bee thoraces were homogenised in 20 ml of distilled water. After filtration and centrifugation the deposit was treated with undiluted lactic acid than examined microscopically at ×100 magnification (Ritter, 1996). Results were compared using the Fisher exact test (Zar, 1974).

2.6. Field observations

Field observations were conducted during summer 2000 (on July and August), in two areas of intensive crop cultures where significant losses in honey bee populations and decrease in honey production had been reported by beekeepers. At the first site, in central France, 7 apiaries were distributed over a 50 square km area around the town of Sens, “département of Yonne” (03°17’7’’ longitude west, 48°11’49.4’’ latitude north). In 5 apiaries, symptoms of trembling and crawling bees evoking chronic paralysis and intermittent abnormal mortalities of several hundred bees were observed in front of several colonies. Two control apiaries without mortality and symptomatic bees were sampled at the first site. At the second location, in the south-west of France, distributed over a 85 square km area around the town of Limoges “départements of Vienne and Haute Vienne”, (01°15’49.5’’ longitude west, 45°50’3.3’’ latitude north) 33 samples were taken from 5 apiaries. Four exhibited abnormal mortalities and irregular signs

similar to those of chronic paralysis and one was an apparently healthy apiary.

Three types of samples were taken for each apiary: dead bees, trembling and crawling bees (sampled outside the hive), and apparently healthy bees collected inside the hive. At least 20 dead bees were sampled for each hive. Twenty and 200 live bees were sampled outside and inside every hive respectively. They were immediately placed at 4 °C and stored at –20 °C before analysis by AGID and RT-PCR tests. Brood frames were observed by visual inspection to detect *Varroa destructor* on bees and cells, and bees with associated symptoms such as deformed wings (DeJong et al., 1982; Bowen Walker et al., 1999). Screening for nosemosis, amœba and acarine mites was also conducted according to previously described diagnostic methods (Ritter, 1996; Steche and Ruijter, 1996). Results were compared using the Fisher exact test (Zar, 1974).

3. RESULTS AND DISCUSSION

The aims of this study were to develop specific tools for diagnosis of CBPV infection in honey bees, whether the infection was latent or accompanied by clinical symptoms, to assess the sensitivity and specificity of this tool, and to demonstrate its practicality for monitoring infection in common apiaries.

3.1. RT-PCR specificity and sensitivity test

RNA extractions provided A260/A280 ratios of 1.9 to 2.4. Visualisation of PCR amplification in agarose gels allowed the detection of one product at 455 bp (Fig. 1).

The new RT-PCR diagnosis tool we developed is based on partial sequence from the putative viral RNA polymerase gene of CBPV. This detection is specific to the CBP virus since the sequence of this putative gene did not reveal homologies greater than 26%, either at the amino acid or nucleic levels, with the other sequences of RNA polymerase genes of bee viruses available in the GenBank database.

Primers were compared to all of the available honey bee virus sequences. The maximum degree of sequence identity was 70% which occurred between CBPV2 primer and BQCV with approximated melting temperature of 45 °C (Wahl et al., 1987). Degrees of sequence identity occurred between CBPV1 primer and BQCV and between CBPV2 primer and ABPV or SBV were 66% with approximated melting temperature between 44 and 46 °C (Wahl et al., 1987).

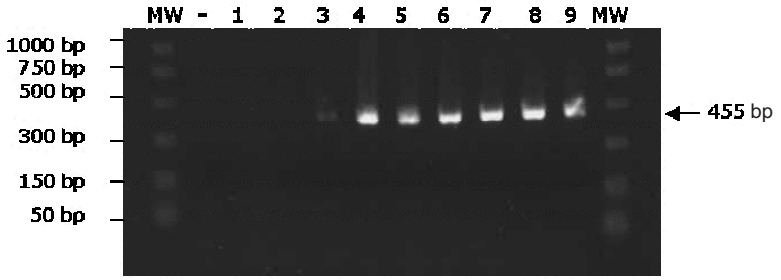

Figure 1. RT-PCR: visualisation of products following agarose gel electrophoresis. Each lane was loaded with 5 µl of PCR product. Lanes MW: PCR molecular weight VIII (Roche). Lane –: negative control with buffer. Lanes 1 to 9: amplification from samples (cf. Tab. I).

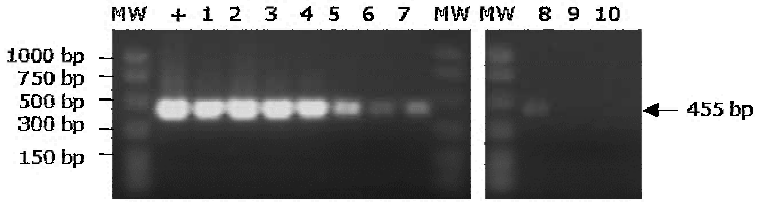

Figure 2. Sensitivity of the RT-PCR test on dilutions of a positive sample from experimentally infected bees: visualisation of RT-PCR products following agarose gel electrophoresis. Each lane was loaded with 8 µl of PCR product. Lane MW: PCR molecular weight (Promega). Lane +: positive control undiluted. Lane 1 to 10: dilutions of the positive control 10^{-1} to 10^{-10}.

The identity of the seven first PCR products (Fig. 1, [AF461053] to [AF461059]) and of two PCR products from field samples of asymptomatic bees ([AF461060] and [AF461061]) was confirmed by sequencing. Sequence similarity was determined using CBPV sequence GenBank [AF375659] as reference. Nucleotide sequence identities ranged from 95 to 100%. Moreover, the identity of the amplification products was confirmed by Southern blotting from ten field samples of asymptomatic bees.

The RT-PCR test applied on 10-fold serial dilutions of a positive extract of experimentally-infected bees was able to detect the virus until it reached the 10^{-8} dilution (Fig. 2).

3.2. Characteristics of three diagnostic tests

Table I presents the results of the three tests on bee samples from hives H1, H2 and H3, before and after experimental infection (EI).

No symptoms or mortalities were observed following experimental infection in bees from one asymptomatic hive (H1), even for the less diluted bee extract (10^{-1}). AGID and RT-PCR tests were negative on the sampled bees from hive1 and on bees inoculated with bee extracts. Thus, this colony H1 was considered uninfected (Allen and Ball, 1996).

Bees from the second asymptomatic hive (H2) developed symptoms following inoculation of the 10^{-3} dilution of bee extract from the same hive. AGID was negative on non-inoculated bees but positive in inoculated ones. A higher dilution (10^{-4}) of this extract or inoculation of physiological saline failed to induce symptoms and to yield a positive AGID test. Therefore, colony H2 was considered to be inapparently or latently infected. For this colony, RT-PCR was positive on non-inoculated bees demonstrating the capacity of this test to detect infection directly on bees sampled from latently infected hive.

The third situation corresponded to a naturally CBPV infected hive 3 with clinical symptoms of chronic paralysis. After experimental inoculation of the 10^{-8} dilution of a H3 bee extract to non-infected bees (H1), symptoms were visible at day 5 post-inoculation and all inoculated bees died at day 7 post-inoculation (Bailey et al., 1963). AGID and RT-PCR tests were positive on non-inoculated and inoculated bees which leads to the classification of this colony as expressing “clinical disease”. Virus purified from experimentally infected bees always induced clinical signs of CBPV infection in the EI test, and gave positive AGID results. The positive RT-PCR test on bees experimentally inoculated with this viral suspension confirmed the specificity

Table I. Evaluation RT-PCR for chronic bee paralysis virus detection in non infected, latently infected and clinical infected hives in comparison to the experimental infection (or infectivity test) and AGID test.

Hive (symptoms)	CBPV status	Samples tested	Results of tests			Sample No in Fig. 1
			EI [a]	AGID	RT-PCR	
H1 (-)	Non infected	H1 bees	ND	-	-	1
		H1 bees inoculated with 10^{-1} H1 bee extract	-	-	-	2
H2 (-)	Inapparent infection	H2 bees	ND	-	+	3
		H2 bees inoculated with 10^{-3} H2 bee extract	+	+	+	4
		H2 bees inoculated with 10^{-4} H2 bee extract	-	-	+	5
		H2 bees inoculated with water	-	-	+	6
H3 (+)	Clinical disease	H3 bees	ND	+	+	7
		H1 bees inoculated with 10^{-8} H3 bee extract	+	+	+	8
		H1 bees inoculated with purified virus	+	+	+	9

[a] EI: experimental infection.
ND: not determined.

of the RT-PCR test designed from purified virus (data not shown).

Results of RT-PCR tests were in total agreement with the EI results for the detection of latent infection (Tab. I) and, in combination with the AGID diagnosis, allowed us to classify the hives according to their CBPV infection status (Tab. I).

3.3. Observations in an experimental apiary

AGID and RT-PCR were used to monitor CBPV infection status of colonies of our experimental apiary. Diagnosis by AGID gave negative results on apparently healthy bees at day one and at the end (11 July and 25 October) of our experiment. Diagnosis by PCR revealed in these same samples that (i) 17 colonies were positive and 3 were negative at these dates, (ii) 9 negative colonies became positive and (iii) 4 positive colonies became negative (Tab. II). During the period of observation, on July and August, corresponding to a high activity of bees, 14 colonies with a lot of mortalities and symptoms were found positive by the AGID test. The RT-PCR test was able to detect CBPV infections in many colonies (Tab. II) which indicated good sensitivity.

Table II. Chronic bee paralysis virus infection follow-up in an experimental apiary (33 hives) between July and October 2000.

11 July		July and August	27 October		Status of hives	Number of colonies
RT-PCR	AGID	AGID	RT-PCR	AGID		
+	-	+	+	-	Latent infection with an episodic clinical disease	8
+	-	-	+	-	Latent infection	9
-	-	+	+	-	Acquired latent infection with an episodic clinical disease	4
-	-	-	+	-	Acquired latent infection	5
-	-	-	-	-	No infection	3
+	-	+	-	-	Recovery after an episodic clinical disease	1
+	-	-	-	-	Recovery after a latent infection	3

In these colonies, little or no *Varroa destructor* and deformed bees were observed on the frames after an attentive examination. All diagnostic tests for acarine mites were negative, and no relationship was established between the presence of nosemosis and the CBPV clinical disease ($P = 0.5$).

The combination of RT-PCR with the AGID diagnosis allowed us to classify the hives according to their CBPV infection status and to monitor the evolution of the infection. AGID was used to confirm the etiology of the clinical disease and RT-PCR was used to reveal latent infection (Tab. II). DNA sequence similarities between the PCR products of two asymptomatic bee samples and the reference were 95 and 97%, respectively, indicating that these results were not false positives. Interestingly, some hives were positive in RT-PCR but did not display clinical disease symptoms, confirming that external (Giauffret, 1968; Allen and Ball, 1996) or genetic factors (Rinderer and Rothenbuhler, 1975; Ball and Bailey, 1997) may influence the onset of a clinical phase.

3.4. Field observations

Table III presents combined results of the field observations. A colony was considered positive in a test when at least one of the two or three samples was positive. In 5 apiaries, more than 50% of colonies had symptoms of trembling and crawling bees, evoking chronic paralysis and intermittent abnormal mortalities with several hundred bees in front of colonies. In every apiary approximately 10% of the hives presented crawling and some dead bees but no trembling bees. A total of 33 hives were sampled: 24 were diagnosed positive in AGID for clinical CP (43 positive samples out of 75), and 31 were diagnosed positive in RT-PCR (69 positive samples out of 75).

Two apiaries without symptom or mortality tested negative with AGID but positive with RT-PCR revealing a latent infection. Hives sampled in the third

Table III. Diagnosis of chronic bee paralysis virus infection in 12 apiaries from field observations using AGID and RT-PCR tests.

Tests	Apiaries		Hives	
	With clinical symptoms and losses	Without clinical symptoms and losses	With clinical symptoms and losses	Without clinical symptoms and losses
AGID+ RT-PCR+	9	0	24	0
AGID– RT-PCR+	0	2	1	6
AGID– RT-PCR–	0	1	0	2
Total	9	3	25	8

control apiary were negative for both tests and considered to be uninfected.

The presence of three other major diseases was observed at both sites: nosemosis, amoebosis and varroasis were present in 8, 6 and 12 apiaries respectively out of 12 apiaries. All diagnostic tests for acariosis were negative. No relationship was established between the presence of these diseases and clinical or latent CBPV infection. Results of Fisher exact test indicate that nosemosis presence and CBPV infection were not significantly related ($P = 0.6$). Presence of varroasis and amoebosis were also independent of CBPV ($P = 1$).

The availability of more sensitive techniques for CBPV detection offers a means for investigating the factors that may induce the acute and latent forms of the disease (Podgwaite and Mazzone, 1986). Bee losses due to chronic paralysis could be related to an imbalance between the number of colonies and forage availability (Allen and Ball, 1996). A "significant positive regression of paralysis outbreaks in England and Wales" was interpreted by authors as a result of relatively increased foraging activity (Bailey et al., 1983). Marked activity induced by an increase in available forage also leads to decrease CBPV dissemination by contacts between infected and healthy individuals within infected colonies (Bailey et al., 1981). In contrast, bad weather conditions in summer increase contaminations within infected colonies. During our field observations, colonies with significant losses were located in two areas of intensive crop cultures, whereas beekeepers reported no spell of rain, wind or cold weather longer than one day during this summer.

Other factors, such as food quality (Podgwaite and Mazzone, 1986), heredity (Bailey, 1965; Rinderer and Rothenbuhler, 1975; Kulincevic and Rothenbuhler, 1989), or anthropogenic factors like chemical contamination in food (Morse, 1997) may influence susceptibility to chronic paralysis and the development of CBPV infections (Podgwaite and Mazzone, 1986; Harvell et al., 1999).

4. CONCLUSION

Reliable diagnostic tests for CBPV will allow researchers to address important questions related to epidemiology of this rather common virus: how is the virus distributed and disseminated in the hives and from the hive to hive? Which factors trigger the development of infection from Type 1 or Type 2 syndrome and determine the severity of losses? For such studies to develop, quantitative PCR may constitute a

significant improvement to investigate how an inapparent viral infection can be transformed into an acute form.

ACKNOWLEDGMENTS

M. RIBIÈRE is a recipient of a fellowship from the « Provence Alpes Côte d'Azur » Regional Council. We are grateful to Dr. N. Tordo (Institut Pasteur, Paris, France) for his support and advice and to Dr. Michel Aubert (AFSSA Sophia Antipolis, France) for valuable comments on the manuscript, review and English advice and methodological assistance. We would also like to thank the Ickowicz company (Bollène, France) for its generous support of this research and the beekeepers who permitted field survey.

Résumé – Diagnostic moléculaire du virus de la paralysie chronique de l'abeille. Le virus de la paralysie chronique (CBPV) est responsable de la paralysie chronique de l'abeille adulte, *Apis mellifera* L. (Bailey, 1968). Cette maladie a été décrite sous deux formes (Ball and Bailey, 1997). La forme classiquement reconnue par les apiculteurs français est la « Maladie Noire » (Giauffret et al., 1967), appelée syndrome de Type 2; elle survient principalement au printemps et au début de l'été avec comme symptômes des abeilles incapables de voler, rampantes et tremblantes, souvent noires, devant les ruches (Faucon, 1992). Le syndrome de Type 1, premièrement décrit en Angleterre et plus récemment reconnu en France, peut subvenir à différentes périodes de l'année et est caractérisé par des abeilles incapables de voler, rampantes et tremblantes avec peu ou pas d'abeilles noires (Allen and Ball, 1996 ; Ball and Bailey, 1997).
Cependant la présence d'abeilles tremblantes et rampantes peut être observée lors d'autres maladies (Mouches et al., 1984 ; Shimanuki, 1997) ou à la suite d'intoxications par des pesticides ou des pollens toxiques (Giauffret and Lambert, 1972 ; Shimanuki, 1997), un diagnostic de laboratoire est donc nécessaire pour déterminer l'étiologie de ces syndromes.
Les infections cliniques par le CBPV s'accompagnent de la production en quantité d'antigènes viraux, elles peuvent donc être détectées par un test en immunodiffusion en gélose (IDG) (Ribière et al., 2000). Cependant, le CBPV persiste dans les colonies sous forme d'infections latentes (Ball, 1996) et dans ce cas la seule méthode de diagnostic disponible jusqu'alors était l'injection d'extraits d'abeilles apparemment saines à des abeilles de la même colonie, ce qui déclenche la maladie observable par la présence de symptômes et de mortalités typiques (Bailey et al., 1963). Ces tests d'infectivité sont longs et fastidieux. Pour ces raisons, nous avons développé un test basé sur la détection de l'ARN viral par RT-PCR comme diagnostic rapide et sensible des infections par le CBPV déclarées et latentes.
Les amorces utilisées en PCR ont été déterminées sur la séquence potentielle de l'ARN polymérase virale (référence GenBank [AF375659]). Les séquences de neufs produits de PCR ont été soumises à la base de donnée GenBank et portent les numéros d'accès [AF461053] à [AF461061]. Ces séquences ont été alignées et comparées à la séquence de référence [AF375659] à l'aide du programme ALIGN (FASTA program version 2.0, W.R. Pearson & University of Virginia, USA). Les pourcentages d'identité variés entre 95 et 100 %. Cette détection est spécifique du CBPV car cette séquence ne présente pas d'homologie dépassant 26 % avec les séquences nucléiques ou protéiques des ARN polymérases potentielles des autres virus de l'abeille disponible sur la base de données GenBank. La spécificité de l'amplification a été confirmée par Southern blot, sur dix échantillons issus de ruches sans symptôme, en utilisant comme sonde le produit de PCR obtenu pour la souche de référence.
Le test par RT-PCR a montré sa capacité de détection du virus sur des dilutions sériées jusqu'à une dilution de 10^{-8} d'extraits d'abeilles infectées expérimentalement (Fig. 2).
Les caractéristiques des trois tests (infection expérimentale, IDG et RT-PCR) ont été

comparées sur des échantillons issus de deux colonies asymptomatiques (Ruche 1 et 2) et d'une colonie symptomatique (Ruche 3) avant et après infection expérimentale. Les résultats du test par RT-PCR (Fig. 1) ont montré une corrélation parfaite avec ceux obtenus en infection expérimentale pour la détection d'une infection latente (Tab. I). Appliqués durant l'été 2000, les tests par RT-PCR et IDG ont permis de classer les 33 colonies de notre rucher expérimental (Tab. II) et les colonies prélevées au cours de deux observations de terrain (Tab. III) en fonction de leur statut face à l'infection par le CBPV comme non infectées, infectées latentes et cliniquement infectées.

Le test par RT-PCR mis au point lors de cette étude s'est révélé très sensible pour la détection des infections inapparentes. Il pourrait par la suite constituer un outil pour des études épidémiologiques de cette maladie.

virus de la paralysie chronique de l'abeille (CBPV) / infection latente / diagnostic RT-PCR / caractérisation du statut CBPV / *Apis mellifera*

Zusammenfassung – Molekularer Nachweis der Infektion mit dem Chronischen Bienenparalyse Virus. Das Virus der chronische Bienenparalyse (CBPV) befällt adulte Bienen (*Apis mellifera)* und führt zur chronischen Paralyse (Bailey, 1965 und 1968), die in 2 von Ball und Bailey (1997) beschriebenen Formen vorkommt. Eine klassische Form, Typ 2 Syndrom, wurde in Frankreich im Frühjahr und frühen Sommer als "maladie noire" beschrieben. Typisch für das Krankheitsbild sind Gruppen mit flugunfähigen, krabbelnden und zitternden Bienen und das Auftreten einiger schwarzer Einzeltiere am Flugloch (Giauffret et al., 1967; Faucon, 1992 und 1996). Das Typ 1 Syndrom, das zuerst in England beschrieben und erst vor kurzem auch in Frankreich bekannt wurde, kommt unabhängig von der Saison vor und es treten zitternde und krabbelnde Bienen aber weniger bzw. keine schwarzen Tiere auf.

Zitternde und krabbelnde Bienen kommen auch bei anderen Bienenkrankheiten (Sanchis und Giauffret, 1972; Mouches et al., 1984; Shimanuki, 1997; Albisetti, 1998) oder als Folge einer akuten oder chronischen Vergiftung mit Pestiziden oder giftigem Pollen vor (Giauffret et al., 1967; Giauffret und Lambert, 1972; Shimanuki, 1997), Daher wird ein Labortest benötigt, um die Ursache dieser Syndrome zu erkennen.

Bei klinischem Auftreten der CBPV Infektionen trat ein hoher Gehalt von viralem Antigen auf, das leicht durch einen Agarose Gel Immunodiffusion Test (AGID) nachgewiesen werden kann (Ribière et al., 2000). CBPV überdauert jedoch in einer Population als unscheinbare latente Infektion (Ball, 1996). Die einzige Diagnosemethode war eine Injektion von Extrakten aus scheinbar gesunden Bienen in adulte Tiere desselben Volks, wodurch die klinischen Symptome der Krankheit und ein Absterben auftreten können (Bailey et al., 1963 und 1981). Diese Infektionstests sind anspruchsvoll und zeitaufwendig. Aus diesem Grund entwickelten wir einen neuen RT-PCR Test zur Erkennung der RNA des Virus als schnelle und empfindliche Diagnose einer Infektion, auch einer latenten, mit dem Chronischen Bienenparalyse Virus.

CBPV PCR Primer wurden entsprechend der Sequenz des putativen viralen RNA Polymerase Gens von CBPV erstellt (GenBank Referenz [AF375659]). Neun Nukleotidsequenzen der PCR Produkte wurden der GenBank Datensammlung unter den Zugriffsnummern [AF461053] bis [AF461061] zugefügt. Sie wurden unter Verwendung des ALIGN Programs (FASTA, Pprogrammversion 2.0, W.R. Pearson & University of Virginia, USA) mit der CBPV-Referenzabstammungslinie [AF375659] verglichen. Die Übereinstimmungen der Nukleotidsequenzen lagen zwischen 95 und 100 %. Diese Erkennung ist spezifisch für CBPV da die Sequenz

dieses putativen Gens weder auf dem Level der Aminosäuren noch dem der Nucleotide Homologien von mehr als 26 % mit Sequenzen des RNA Polymerase Gens von anderen in der GenBank gespeicherten Bienenviren aufzeigt.

CBPV PCR Primer wurden auf Grund der Sequenz des vermeintlichen RNA Polymerase Gens des CBP Virus gebildet (nicht publizierte Daten, GenBank Referenz [AF375659]) und es ergab keine Identität der Sequenz mit vorhandenen Sequenzen von anderen Bienenviren. Die Identität von den 7 ersten PCR Produkten (Abb. 1) wurde durch Sequenzierung bestätigt. Zusätzlich erfolgte eine Bestätigung durch die Identität der Amplifikationsprodukte von Bienen ohne Symptome aus 10 Feldproben durch Southern Blotting. Die Anwendung des RT-PCR Test bei 10maliger Serienverdünnung eines positiven Extrakts von experimentell infizierten Bienen erwies sich als ausreichend empfindlich, um Viren bis zu einer 10^{-8} - fachen Verdünnung nachzuweisen (Abb. 2).

Die Leistungsfähigkeit wurde in 3 Tests (experimentelle Infektion, AGID und RT-PCR) durch den Vergleich mit Proben von 2 Völkern ohne Symptome (Volk 1 und 2) und einem Volk mit Symptomen (Volk 3) ermittelt, und zwar vor und nach einer experimentellen Infektion. Die Ergebnisse des RT-PCR Tests (Abb. 1) ergaben eine gute Korrelation mit den Ergebnissen der experimentellen Infektion für die Erkennung einer latenten Infektion (Tab. I). Die Methode wurde im Sommer 2000 bei 33 Völkern an unserem Versuchsbienenstand (Tab. II) und bei 2 Feldversuchen (Tab. III) überprüft. Der RT-PCR Test in Kombination mit der AGID Diagnose ermöglichte die Charakterisierung des CBPV Status der Völker als nicht infiziert, latent infiziert und infiziert mit Auftreten von klinischen Symptomen (Tab. II und III).

Der hier beschriebene RT-PCR Test erwies sich als hoch empfindlich zur Entdeckung von unterschwelligen Infektionen. Er könnte sich als nützliches Instrument erweisen, um die Epidemiologie dieser Krankheit zu untersuchen.

Chronischer Bienenparalyse Virus (CBPV) / latente Infektion / RT-PCR Diagnose / Charakterisierung des CBPV Status / *Apis mellifera*

REFERENCES

Albisetti J. (1998) Un groupe d'agents pathogènes de l'abeille *Spiroplasma Apis*, Bull. Tech. Apic. 25, 21–25.

Allen M., Ball B.V. (1996) The incidence and world distribution of honey bee viruses, Bee World 77, 141–162.

Anderson D.L., Gibbs A.J. (1988) Inapparent Virus Infections in Pupae of Honey Bee (*Apis mellifera* Linnaeus) in Australia, J. Gen. Virol. 69, 1617–1625.

Bailey L. (1965) Paralysis of Honey Bee, *Apis mellifera* Linnaeus, J. Invertebr. Pathol. 7, 132–140.

Bailey L. (1968) The purification and properties of chronic bee-paralysis virus, J. Gen. Virol. 2, 251–260.

Bailey L., Ball B.V., Perry J.N. (1981) The prevalence of viruses of honey bees in Britain, Ann. Appl. Biol. 97, 109–118.

Bailey L., Ball B.V., Perry J.N. (1983) Honeybee paralysis: its natural spread and its diminished incidence in England and Wales, J. Apic. Res. 22, 191–195.

Bailey L., Gibbs A.J., Woods R.D. (1963) Two viruses from adult honey bees (*Apis mellifera* Linnaeus), Virology 21, 390–395.

Bailey L., Woods R.D. (1977) Two more small RNA viruses from honey bees and further observations on sacbrood and acute bee-paralysis viruses, J. Gen. Virol. 37, 175–182.

Ball B.V. (1996) Honey bee viruses: a cause for concern?, Bee World 77, 117–119.

Ball B.V., Bailey L. (1991) Viruses of honey bees, in: Adams J.R., Bonami J.R. (Eds.), Atlas of invertebrate viruses, CRC Press, Boca Raton, USA, pp. 525–551.

Ball B.V., Bailey L. (1997) Viruses, in: Morse R.A., Flottum K. (Eds.), Honey bee pests, predators & diseases, A.I. Root Company, Medina, pp. 11–32.

BowenWalker P.L., Martin S.J., Gunn A. (1999) The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud, J. Invertebr. Pathol. 73, 101–106.

DeJong D., DeJong P.H., Gonçalves L.S. (1982) Weight loss and other damage to developing

worker honeybees from infestation with *Varroa jacobsoni*, J. Apic. Res. 21, 165–167.

Faucon J.P. (1992) Paralysie chronique ou maladie noire, in: CNEVA-FNOSAD (Ed.), Précis de Pathologie : connaître et traiter les maladies des abeilles, CNEVA-FNOSAD, pp. 204–212.

Faucon J.P. (1996) La question sanitaire, FNOSAD, Angers.

Giauffret A. (1968) Les viroses de l'abeille, Rev. Pathol. Comp. 111–115.

Giauffret A., Caucat M.J., Amargier A., Bres N. (1967) Étude histopathologique de la maladie noire de l'abeille, Bull. Apic. X, 133–144.

Giauffret A., Lambert M. (1972) Le diagnostic de la maladie noire de l'abeille, Recl. Méd. Vét. CXLVIII, 869–877.

Harvell C.D., Kim K., Burkholder J.M., Colwell R.R., Epstein P.R., Grimes D.J.R., Hofmann E.E., Lipp E.K., Osterhaus A.D., Overstreet R.M., Porter J.W., Smith G.W., Vasta G.R. (1999) Emerging marine diseases: climate links and anthropogenic factors, Science 285, 1505–1510.

Huang X., Miller W. (1991) A time-efficient, linear-space local similarity algorithm, Adv. Appl. Math. 12, 337–357.

Kulincevic J.M., Rothenbuhler W.C. (1989) The effects of artificial infection with chronic bee paralysis virus on queens from strains of honeybees resistant or susceptible to hairless-black syndrome, J. Apic. Res. 28, 79–80.

Morse R.A. (1997) Honey bees and pesticides, in: Morse R.A., Flottum K. (Eds.), Honey Bee Pests, Predators and Diseases, A.I. Root Company, Medina, pp. 551–549.

Mouches C., Bové J.M., Albisetti J. (1984) Pathogenicity of *Spiroplasma apis* and other spiroplasmas for honey-bees in southwestern France, Ann. Microbiol. 135, 151–155.

Myers E.W., Miller W. (1988) Optimal alignments in linear space, Comput. Appl. Biosci. JID-8511758 4, 11–17.

O.I.E. (1996) Bees, in: O.I.E. Standards Commission (Ed.), Manual of Standards for Diagnostic Tests and Vaccines, Paris, Office International des Epi zooties, pp. 598–617. 2001 version available at: http://www.oie.int/eng/normes/mmanual/A_00102.htm (verified 14 March 2002).

Podgwaite J.D., Mazzone H.M. (1986) Latency of insect viruses, Adv. Virus Res. 31, 293–320.

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

Rinderer T.E., Rothenbuhler W.C. (1975) Responses of three genetically different stocks of the honeybee to a virus from bees with hairless-black syndrome, J. Invertebr. Pathol. 25, 297–300.

Ritter W. (1996) Acariosis of bees, in: O.I.E. Standards Commission (Ed.), Manual of standards for Diagnostic Tests and Vaccines, Paris, Office International des Epizooties, pp. 599–602. 2001 version available at: http://www.oie.int/eng/normes/mmanual/A 00102.htm (verified 14 March 2002).

Sambrook J., Fritsch E.F., Maniatis T. (1989) Molecular Cloning. A laboratory manual, Cold Spring Harbor Laboratory Press, New York.

Sanchis R., Giauffret A. (1972) La rickettsiose de l'abeille, Bull. Apic. 15.

Shimanuki H. (1997) Synonymy in bee disease, in: Morse R.A., Flottum K. (Ed.), Honey Bee Pests, Predators and Diseases, A.I. Root Company, Medina, pp. 534–537.

Steche W., Ruijter A. (1996) Nosemosis of bees, in: O.I.E. Standards Commission (Ed.), Manual of standards for Diagnostic Tests and Vaccines, Paris, Office International des Epizooties, pp. 611–613. 2001 version available at: http://www.oie.int/eng/normes/mmanual/A00102.htm (verified 14 March 2002).

Wahl G.M., Berger S.L., Kimmel A.R. (1987) Molecular hybridization of immobilized nucleic acids: Theoretic concepts in pratical conciderations, Methods Enzymol. 152, 399–407.

Zar H.J. (1974) Biostatistical Analysis, Prentice-Hall, Englewood Cliffs, NJ, USA.