Available online at www.sciencedirect.com
ScienceDirect
Journal of Virological Methods 147 (2008) 275–281
Journal of Virological Methods
www.elsevier.com/locate/jviromet

A sensitive one-step real-time RT-PCR method for detection of deformed wing virus and black queen cell virus in honeybee *Apis mellifera*

Deborah Kukielka [a], Fernando Esperón [a], Mariano Higes [b], Jose Manuel Sánchez-Vizcaíno [a,*]

[a] *Animal Health Department, Faculty of Veterinary Science, Universidad Complutense de Madrid, Avda/Puerta de Hierro s/n, 28040 Madrid, Spain*
[b] *Centro Apícola Regional, Junta de Comunidades de Castilla La Mancha, Guadalajara, Spain*

Received 26 June 2007; received in revised form 11 September 2007; accepted 17 September 2007
Available online 26 October 2007

Abstract

A one-step real-time RT-PCR based on SYBR Green (SG) chemistry was developed for the detection, differentiation and quantification of two of the most common viruses on the honeybee *Apis mellifera* L., deformed wing virus and black queen cell virus. Two sets of primers specific for each virus, were designed in conserved regions of the viral genome for their use in the one-step real-time RT-PCR. Both reactions were optimized for highest sensitivity and specificity and SG-based real-time was used to achieve quantitative detection. All samples evaluated in this study were from Spanish honeybee colonies. Viral detection and identification was confirmed by sequencing of the PCR products. The described one-step real-time SG RT-PCR proved to be a fast, accurate and useful technique to detect and even quantify these honeybee viruses that cause unapparent infections, and might contribute with other factors to the increasing honeybee colonies depopulation.

Keywords: Real-time RT-PCR; SYBR Green; Honeybee; Depopulation; BQCV; DWV

1. Introduction

The honeybee *Apis mellifera* is subject of many viral infections that have been increasingly investigated during the last decade. In general, honeybee viruses are widespread and most of them cause unapparent and persistent infections (Bailey et al., 1981; Evans and Hung, 2000; Hung and Shimanuki, 1999), and environmental factors or parasite infestations may activate virus infections and sometimes, lead to the appearance of clinical symptoms. It is usually accepted that some viruses interact with *Varroa destructor*, in a multi-factorial complex named "parasitic mite syndrome" (Shimanuki et al., 1994). Important and frequent viral infections include: acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), sacbrood virus (SBV), Kashmir bee virus (KBV), deformed wing virus (DWV) and black queen cell virus (BQCV). These viruses affect honeybees and their production causing important economic looses due to their reduced honey production and population decrease since honeybees are commonly used to pollinate crops, reaching an annual market value around 15 billion dollars in the USA (Morse and Calderon, 2000).

BQCV and DWV are thought to participate in some bee colony collapses that are increasingly occurring in most of the honey producer countries in Europe and the United States (Chen et al., 2005). Black queen cell virus (BQCV), previously part of the "picorna-like" group of viruses affecting honeybees (Leat et al., 2000), has recently been classified within the new genus *Cripavirus* (family *Dicistroviridae*) (Mayo, 2002). BQCV has a monopartite bicistronic genome with non-structural genes at the 5′ end and structural genes at the 3′ end. It causes mortality particularly in queen pupae and pupae, but it is also common in adult bees specially in spring and early summer (Berenyi et al., 2006), and is considered to be triggered by *Nosema apis* infestation (Allen and Ball, 1996; Bailey and Woods, 1974; Bailey et al., 1983). Deformed wing virus (DWV) is a picorna-like virus belonging to the genus *Iflavirus* (Mayo, 2002). DWV has a monopartite monocistronic genome with structural genes at the 5′ end and non-structural genes at the 3′ end. Sometimes it produces no clinical signs of infection while other times it causes wing deformities, bloated abdomens, paralysis and a shorter

* Corresponding author. Tel.: +34 91 394 40 82; fax: +34 91 394 39 08.
E-mail address: jmvizcaino@vet.ucm.es (J.M. Sánchez-Vizcaíno).

0166-0934/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2007.09.008

adult life span for emerging worker and drone bees. It is associated with *V. destructor* parasitation (Bowen-Walker et al., 1999; Nordstrom, 2003).

The study of the virus affecting honeybees lacks of data related to the real role of these viruses in the health status of the beehives. Diagnosis of bee virus infections is difficult because these viruses usually persist as in apparent infections and cause no overt signs of disease (Bailey, 1967). The classical diagnostic methods for the detection of honeybee viruses are based on electron microscopy and serological methods, which have the drawbacks of limited availability of antisera and problems with the specificity of some antisera as a result of antiserum production from preparations containing virus mixtures (Benjeddou et al., 2001).

The use of a specific probe-based assay such as TaqMan real-time PCR requires high complementarity for probe binding, which might result in a failure to detect high sequence variability in the probe-binding region. Non-specific assays using intercalating dyes such as SYBR Green I are found to be more reliable, flexible, simple, and of lower cost for detecting nucleic acid targets characterized by sequence variability, compared with TaqMan real-time PCR, especially for RNA viruses. SYBR Green I is a minor groove DNA binding dye with a high affinity for double-strand DNA (dsDNA) and exhibits fluorescence enhancement upon binding to dsDNA. The accumulation of amplified DNA is measured by the increase in fluorescence over time, followed by confirmation of results by melting curve analysis (Heid et al., 1996).

The SYBR Green-based real-time RT-PCR is an excellent alternative for the detection of viruses in honeybees because of its simplicity and price. Several RT-PCR methods have been developed and applied for the diagnosis of so far seven bee viral infections (Bakonyi et al., 2002; Benjeddou et al., 2001; Blanchard et al., 2007; Chen et al., 2005; Grabenstiner et al., 2001; Ribière et al., 2002; Stoltz et al., 1995; Tentcheva et al., 2004; Topley et al., 2005; Genersch, 2005) but no SYBR Green-based techniques have been reported. This work describes the development and evaluation of a single-step real-time RT-PCR based on SYBR Green for the detection and differentiation of two important viruses of honeybees: DWV and BQCV.

2. Materials and methods

2.1. Sampling, samples preparation and RNA extraction

To achieve the best optimization of the reaction, the real-time RT-PCR method was initially carried out with previously positive sequenced controls for DWV and BQCV, including samples that were positive to both viral infections (viral co-infection). These control samples have their origin on the field, were taken among Spanish beehives between 2003 and 2005. They were detected as positive samples using the conventional RT-PCR and were later sequenced. They were only used for the assay optimization and are not included in the general study. Negative samples to both RT-PCR were used in these assays. They were selected after performing repeated extraction procedures and conventional RT-PCR obtaining always negative results. Once the reaction was optimized with the positive and negative controls, 100 honeybee samples from different apiaries distributed along Spain were chosen for evaluation. The distribution of these apiaries is shown in Fig. 1. A random selection of the samples was done with the aim of evaluating the prevalence of these honeybee viruses. The beehives did not show any clinical signs. All samples were collected during springtime. Positive samples to other honeybee viruses (KBV) are also included. The rest of honeybee viruses were not tested in the assays, as we do not have positive controls.

Fig. 1. Distribution and number of samples studied.

Honeybees were taken from the hives and were frozen immediately at −20 °C until assay. For the homogenization, the honeybees were crushed in a mortar with PBS solution in proportion of 10 bees per 5 mL of PBS. After homogenization, the samples were centrifuged for 15 min at 1500 rpm and supernatant was collected. These samples were stored at −80 °C. Each pool of 10 bees was from a different beehive, having samples from 100 different beehives. None of these samples were used for assay optimization.

For RNA extraction, the homogenized samples were kept in sterile eppendorf tubes and were treated with the TRIzol reagent following the manufactures' instructions. Total RNA was resuspended in free RNases and DNases water and maintained at −20 °C.

2.2. Primer design and quantitative real-time PCR protocol by SYBR Green assay

Both pairs of primers were designed to amplify conserved regions of DWV and BQCV viral genomes. For DWV the polyprotein gene was selected and for the BQCV the structural polyprotein gene was selected. Their design was based on different sequences previously published in the GenBank database (accession numbers DQ385507, DQ385505, DQ38502, NC003784) and using Primer Express and Primer 3 software. Both sets of primers were chosen according to their annealing temperatures as they could share the same ThermoCycler Program. The primers were synthesized by Eurogentec and are shown in Table 1.

Table 1
Sequences of the primers and their position in the genome

	Sequence (5′–3′)	Position on the genome	Length of amplified product
DWV2 Fwd	CTG TAT GTG GTG TGC CTG GT	8575	226
DWV2Rev	TTC AAA CAA TCC GTG AAT ATA GTG T	8801	
BQCV3Fwd	AAG GGT GTG GAT TTC GTC AG	7933	284
BQCV3Rev	GGC GTA CCG ATA AAG ATG GA	8217	

A one-step real-time RT-PCR was developed based on SYBR Green detection. All reactions were performed in a Stratagene MxPro 3000 using the Master mix and enzymes provided by Stratagene.

Titration of primers was achieved by using a matrix of reciprocal concentrations (from 100 nM to 900 nM), choosing those that yielded the higher sensitivity and the minimum primer dimmer. To determine their annealing temperature we performed a gradient RT-PCR and chose the best temperature for both set of primers to be able to use them in the same ThermoCycler Program.

The final procedure was performed in a final volume of 25 μL as follows: 2 μL of RNA template, 12.5 μL of SYBR Green Master mix (Quantitative RT-PCR Brilliant SYBR Green Master Mix, Stratagene®), 0.0625 μL of StrataScript RT/Rnase Block Enzyme Mixture (Stratagene®), 0.3 μM of BQCV primers for BQCV detection or 0.2 μM of DWV primers for DWV detection.

The same PCR program for both pair of primers was used. The thermocycler program was: 30 min at 48 °C (RT) followed by 10 min at 95 °C, and 40 amplification cycles of 95 °C for 45 s, 61 °C for 1 min and 72 °C for 1 min. The final step was carried out at 72 °C for 7 min of final extension. Lastly, the dissociation curve was obtained starting at 70–99 °C (40 cycles with 1 °C of increment each 30 s). Both, the DWV and the BQCV amplicons have a T_m of 78 °C (data not shown). Ten microliters of RT-PCR products were analyzed by 2% agarose gel electrophoresis. The expected size of the amplicons was: 226 bp for DWV and 284 bp for BQCV.

2.3. Standard curve

Since honeybee viruses do not grow in cell lines, a standard curve with two high positive field samples for each virus confirmed by the conventional RT-PCRs used as reference (Tentcheva et al., 2004; Benjeddou et al., 2001) and by sequencing was constructed. These two samples were selected when, during the analysis of the 100 field samples, they yielded a really early Ct indicating that their viral load was high. Once these samples were selected, conventional RT-PCR, real-time RT-PCR and sequencing were done. Their viral load was estimated by spectrophotometry. Standard curves were generated with the cDNA amplicon from the real-time RT-PCR performed with these two high samples (Beuve et al., 2007).

Briefly, serial 10-fold dilutions of these two samples were made with the initial amount of the cDNA amplicon obtained from a previous real-time RT-PCR and a later purification. The next step was to quantify these products by spectrophotometry in a Nanodrop and deduce the number of copies from the molecular weight of the sequence of the amplicon (Beuve et al., 2007). The complete sequence of the amplicon was obtained by sequencing with the forward and reverse primers. The amplicon's molecular weight was calculated. The absorbance of the purified product from the SG RT-PCR was divided by the amplicon's molecular weight obtaining the estimated amplicon copies number. This calculation provides an indirect estimation of the number of copies of RNA molecules since the efficiency of RT and RNA extraction were not determined and it allows relative quantification between samples and experiments.

The standard curve was also used in assays with several field samples to study the variability in viral load by extrapolation of their Ct values.

2.4. Specificity, sensitivity, reproducibility of the one-step real-time RT-PCR

The efficacy of the method was assessed by testing the presence of the viruses on a 100 field samples. Results were compared with the sensitivity of the conventional RT-PCR described previously for DWV (Tentcheva et al., 2004) and BQCV (Benjeddou et al., 2001).

The specificity of the RT-PCR assay was evaluated by sequence analysis of size specific amplification products. The RT-PCR products were purified using QIAquick PCR purification Kit (Qiagen) and analyzed by the sequencing services of SECUGEN S.A. (Madrid, Spain). The sequence data of each virus fragment was analyzed using the BLAST® server at the NCBI. Due to the difficulty to obtain positive reference samples for honeybee viruses other than KBV, BQCV and DWV, the specificity of these primers for the rest of the main viral honeybee infections was confirmed using BLAST® and Culstal W® software.

The sensitivity of the method was investigated by testing 10-fold dilutions of two positive field samples for DWV and BQCV and comparing them to the results of the conventional RT-PCR. The same samples were proved with the conventional and the SG RT-PCR, determining their different detection limit.

Reproducibility was demonstrated by evaluating the intra- and inter-assay variability of the Ct values obtained after amplification of 10-fold serial dilutions of the cDNA amplicon from the positive reference samples ranging from 10^{-2} to 10^{-8}. Intra-assay reproducibility was evaluated by analysing three replicates of the 10-fold serial dilutions during the same experiment. The inter-assay reproducibility was estimated by testing serial dilutions of the same positive control in three independent

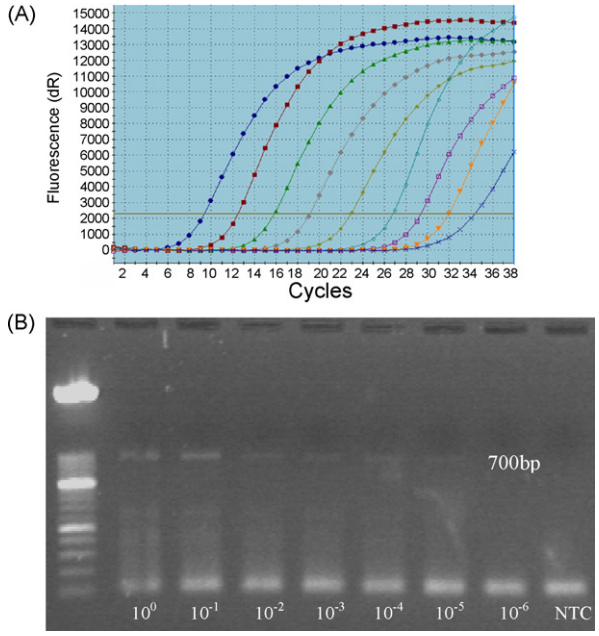

Fig. 2. Comparison of the sensitivities of SG RT-PCR and conventional RT-PCR for BQCV. (A) Amplification plots obtained using serial 10-fold dilutions of BQCV positive sample represented from dilution 10^{-2} to 10^{-9}. Last blue line: NTC. (B) Agarose gel electrophoretic analysis of conventional BQCV RT-PCR product and dilutions from 10^{0} to 10^{-6} of the same positive sample used for SG RT-PCR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

experiments, performed on different days. Variability was estimated by the coefficient of variation (CV) of the mean Ct values obtained from the standard curve.

2.4.1. Conventional RT-PCR

Both conventional RT-PCR for DWV and BQCV were performed based on the protocols described in the reference articles (Tentcheva et al., 2004; Benjeddou et al., 2001). The new primers designed for real-time RT-PCR were also tested in a conventional RT-PCR with the same samples used in the other assays.

3. Results

Both RT-PCR were carried out first with positive sequenced controls that were not included in the final results for the general study. Once the RT-PCRs were developed and optimized, a total of a hundred samples were evaluated for presence of BQCV and DWV. Both viral infections were detected. Time was saved by performing both RT-PCRs with the same thermocycler program in two different mixes.

3.1. Specificity, sensitivity, reproducibility of the one-step real-time RT-PCR

Sequence alignment of the amplification fragments of each virus with published sequences at the GeneBank resulted in a sequence identity between 90% and 100% for both viruses. No amplification occurred with positives samples to KBV. The specificity of these primers for the rest of the main honeybee viral infections (SBD, ABPV and CBPV) was demonstrated by using BLAST® and Culstal W® software (data not shown).

The detection limit was determined by the lowest dilution that showed specific amplification for the strong positive field samples selected as templates. It was 10^{-9} for both qRT-PCR, whereas the conventional RT-PCR detection limit was 10^{-5} for BQCV and 10^{-6} for DWV (Figs. 2 and 3). The conventional RT-PCR was also performed with the new real-time primers. The same strong samples and their dilutions were used to perform these assays. The detection limit for these assays was: 10^{-6} for BQCV and 10^{-5} for DWV (data not shown). The amplicon detection limit was 30.8 amplicons/μL for BQCV and was 30.5 amplicons/μL for DWV when using qRT-PCR. The amplicon detection limit/μL for the conventional RT-PCR with already published primers was 308.000 for BQCV and 30.500 for DWV.

For DWV qRT-PCR, the CV ranged from 0.1% to 3% within a run (intra-assay reproducibility) and from 0.1% to 3.4% from run to run (inter-assay reproducibility); for BQCV qRT-PCR the CV ranged from 1% to 3% within a run (intra-assay reproducibility) and from 0.6% to 4% from run to run (inter-assay reproducibility).

3.2. Standard curve

The number of amplicon copies was determined dividing the absorbance of the purified product from the SG RT-PCR by the molecular weight of the amplicon.

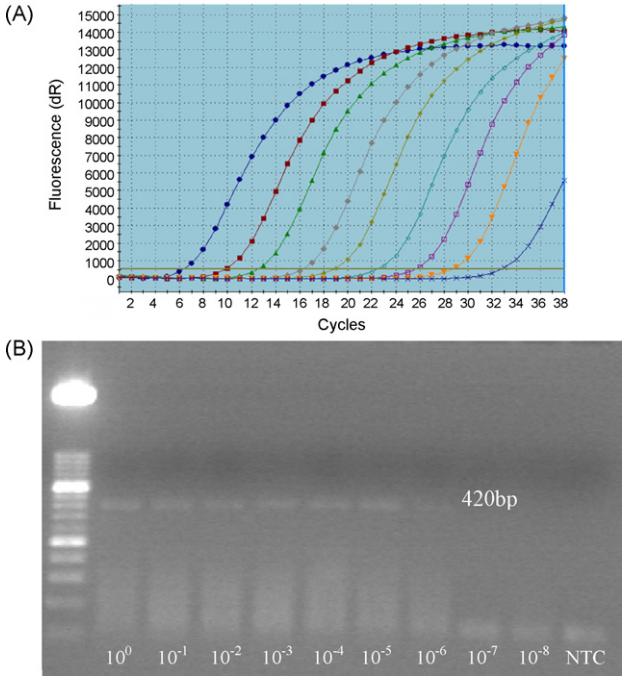

Fig. 3. Comparison of the sensitivities of SG RT-PCR and conventional RT-PCR for BQCV. (A) Amplification plots obtained using serial 10-fold dilutions of DWV positive sample represented from dilution 10^{-2} to 10^{-9}. Last blue line: NTC. (B) Agarose gel electrophoretic analysis of conventional DWV RT-PCR product and dilutions from 10^{0} to 10^{-8} of the same positive sample used for SG RT-PCR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

One microliter of stock cDNA contained 3.084×10^{10} copies/μL for BQCV and 3.05×10^{10} copies/μL for DWV. We plotted the log starting quantity versus the Ct. The Cts were linearly proportional to the logarithm of the input copy number. For DWV, the slope ($S = -3.19$) of the linear regression curve correlated with the efficiency of the PCR reaction ($E = 105.8\%$), according to the formula: $E = (10^{-1/\text{slope}}) - 1$. The R^2 was 0.99. For BQCV, the slope ($S = -3.32$) of the linear regression curve correlated with the efficiency ($E = 100\%$) of the PCR reaction according to the formula: $E = (10^{-1/\text{slope}}) - 1$. The R^2 was 0.99 (Fig. 4). The dilutions were maintained in aliquots to perform the standard curve in different assays.

3.3. Conventional RT-PCR versus real-time RT-PCR

The sensitivity of the method was tested over a hundred samples versus the conventional RT-PCR used as reference. The samples that were positive to the real-time RT-PCR were confirmed by sequencing. Both RT-PCR presented different sensitivity. Results are shown in Table 2.

As can be observed, a higher number of positive (sequenced) samples were detected with the Real-time method (Table 2). The samples that were positive were sequenced confirming the specificity of these methods. The conventional RT-PCR had a sensitivity of 34% for DWV and of 10% for BQCV related to the real-time RT-PCR. The specificity was of 100% for both assays.

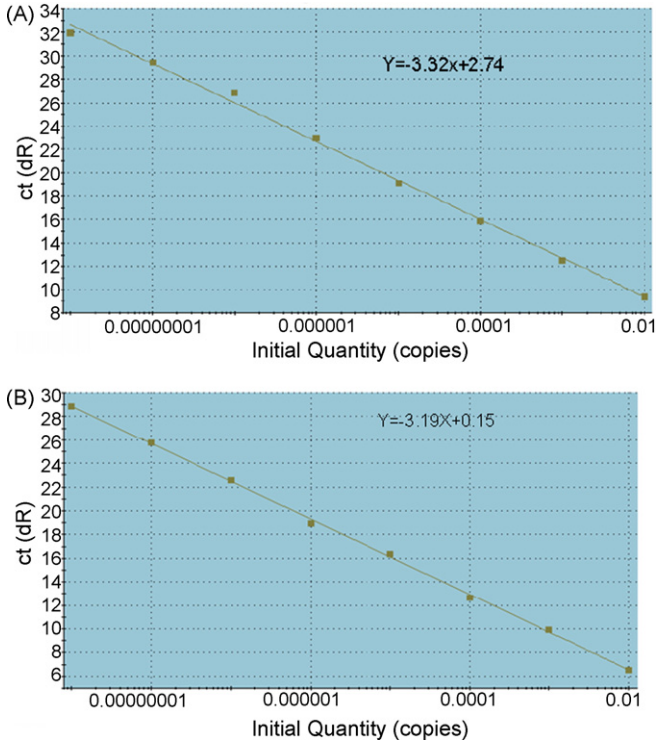

Fig. 4. (A) Standard curve obtained by plotting the Ct value vs. the concentration of amplicon copies of BQCV. (B) Standard curve obtained by plotting the Ct value vs. the concentration of amplicon copies of DWV.

Table 2
Compared sensitivity and specificity between conventional and real-time RT-PCR

	Deformed wing virus		
	Positive	Negative	Total
Sequenced samples			
Real-time[a]			
Positive	79	0	79
Negative	0	21	21
Total	79	21	100
Conventional[b]			
Positive	27	0	27
Negative	52	21	73
Total	79	21	100
	Black queen cell virus		
	Positive	Negative	Total
Sequenced samples			
Real-time[c]			
Positive	76	0	76
Negative	0	24	24
Total	76	24	100
Conventional[d]			
Positive	8	0	8
Negative	68	24	92
Total	76	24	100

[a] Sensitivity 100%, specificity 100%.
[b] Sensitivity 34%, specificity 100%.
[c] Sensitivity 34%, specificity 100%.
[d] Sensitivity 10%, specificity 100%.

4. Discussion

In this study, two one-step real-time RT-PCR were developed to detect the presence of BQCV and DWV infection. The standard curves generated from positive control cDNA of the BQCV and the DWV genome showed that quantification of these genomes was linear over 9 orders of magnitude. The efficiency of both standard curves and their good correlation was confirmed.

In the last few years, the diagnostic methods for honeybee viruses changed from serological to PCR-based methods (Bakonyi et al., 2002; Benjeddou et al., 2001; Blanchard et al., 2007; Chen et al., 2005; Grabenstiner et al., 2001; Ribière et al., 2002; Stoltz et al., 1995; Tentcheva et al., 2004; Topley et al., 2005; Genersch, 2005). These kinds of assay are easily adaptable to simple diagnostic laboratories and due to standard methods are comparable. Moreover, with qRT-PCR we are able to detect and quantify low viral loads, and therefore, persistent unapparent viral infections, which are common in honeybees.

Several RT-PCR techniques have been reported before, but no RT-PCR based on SYBR Green has been described. In this study, the development of two SG real-time RT-PCRs to detect DWV and BQCV, respectively, are described. SYBR Green RT-PCR is more accurate than conventional RT-PCR as they let us to analyze the amplification of our target when the RT-PCR has the highest efficiency. They also allow for quantification of viral loads and have higher sensitivity.

As SYBR Green binds unspecific to any double-stranded DNA, the principal challenge in developing SYBR Green Real-time PCR is choosing the right primers to overcome the primer dimer formation and therefore, the background fluorescence that interferes with the real positive results. We designed one pair of primers to amplify a fragment of the polyprotein gene of DWV and another pair to amplify the non-structural polyprotein gene of the BQCV. The DWV pair yields a fragment of 226 bp and the BQCV pair yields a fragment of 284 bp. Both are specific and their specificity was confirmed by sequencing. Performing both PCR with the same PCR thermocycler program in two different mixes saves considerable time.

The SG-based real-time RT-PCR presented here are more sensitive than the conventional RT-PCR, which have an amplicon detection limit/μL of 308.000 copies for BQCV and 30.500 for DWV. With the SG-based real-time RT-PCR, BQCV has a detection limit of 30.8 copies/μL, and DWV 30.5 copies/μL. These are comparable to the quantification limit reported for a SG qRT-PCR assay in Dengue virus 2 detection (Richardson et al., 2006). Therefore, these techniques are able to detect cases of infection more accurately than the conventional RT-PCR used as reference. In our laboratory and using the same field samples, conventional RT-PCR had a dilution detection limit of 10^{-5} for BQCV and 10^{-6} for DWV while with the new SG-based real-time RT-PCR the dilution detection limit was 10^{-9} for both qRT-PCR. When the new primers were tested on the conventional RT-PCR, their dilution detection limit was 10^{-6} for BQCV and 10^{-5} for DWV (data not shown). Using the real-time RT-PCR both viral infections were detected, even in samples with a very low viral load, as is shown by the detection limit of the real-time RT-PCR.

This high sensitivity and the ability to estimate the number of amplicon copies present in the initial sample allows the detection of multiple viral infections that do not show obvious pathological symptoms, avoiding wrong diagnoses. This fact is supported by our results, since the field samples were collected randomly and not based on the presence of symptoms. The majority of these samples, when analyzed, were positive to one or both of the studied viral infections.

The specificity of these two real-time RT-PCR methods is important to differentiate the coexistent virus that may be present in the same honeybee. No amplification occurred with positive samples to KBV or negative samples. DWV is considered the most widespread virus in Europe (Martin, 2001) and its presence is often associated with *V. destructor* infestation, and the role of this parasite in the transmission of the virus has already been demonstrated. BQCV is the second most prevalent virus in France where a study about the prevalence of six honeybee virus was carried out (Tentcheva et al., 2004). Mixed infections in honeybees are quite common in nature indeed, multiple viruses can be detected in a single bee or different viruses in individual bees of the same colonies (Chen et al., 2004). Co-infection by DWV and BQCV seems very frequent among colonies with virus infections in France (Chen et al., 2004). The evaluation of a hundred field samples in this study yields similar results in Spain. Thus, these RT-PCR assays appear suitable for follow-up studies about viruses' prevalence in honeybee colonies. The results obtained in this study show that the prevalence for DWV is about 79% and about 76% for BQCV when performing the analysis with the real-time SG RT-PCR. As can be observed in our results, the prevalence of these viruses in Spain is high. The next step is to analyze a larger number of samples with these techniques and determine the presence and distribution of these infections among all the Spanish beehives.

The quantitation used in this study has been previously described and demonstrated as useful to estimate the number of copies for viruses that do not grow in cell lines (Beuve et al., 2007). Here, we were able to quantify the amplicon number of copies present in the samples. Considering a constant RT efficiency and by extrapolation from the standard curve we estimated the viral load of the field sample which led us to a better understanding of its meaning in the presence/absence of clinical signs. Since the pathological importance of the multiple infections is still unknown, this kind of quantitative molecular techniques will contribute to increase our knowledge of the relationships between the estimated viral load and the clinical symptoms and to a better understanding of the relation between the virus, the honeybee and their environment. Lastly, these molecular techniques are time saving and reduce diagnostic costs, helping to develop rapid diagnosis strategies and epidemiological studies of bee viral infections.

Acknowledgments

This study is supported by projects API06-005.C2 from the MAPA (Ministerio de Agricultura Pesca y Alimentación) and INIA-RTA2005-164 from INIA (Instituto Nacional de Investigaciones Agrarias).

We gratefully acknowledge Belen Rivera y Rocio Sanchez for their excellent technical assistance.

References

Allen, M., Ball, B.V., 1996. The incidence and world distribution of the bees. J. Gen. Virol. 81, 2111–2119.

Bailey, L., 1967. The incidence of virus diseases in the honeybee. Ann. Appl. Biol. 60 (1), 43–48.

Bailey, L., Woods, R.D., 1974. Three previously undescribed viruses from the honey bee. J. Gen. Virol. 25 (2), 175–186.

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

Bailey, L., Ball, B.V., Perry, J.N., 1983. Association of viruses with two protozoal pathogens of the honey bee. Ann. Appl. Biol. 103, 13–20.

Bakonyi, T., Grabensteiner, E., Kolodziejek, J., Rusvai, M., Topolska, G., Ritter, W., Nowotny, N., 2002. Phylogenetic analysis of acute bee paralysis virus strains. Appl. Environ. Microbiol. 68 (12), 6446–6450.

Benjeddou, M., Leat, N., Allsopp, M., Davison, S., 2001. Detection of acute bee paralysis virus and black queen cell virus from honeybees by reverse transcriptase PCR. Appl. Environ. Microbiol. 67 (5), 2384–2387.

Berenyi, O., Bakonyi, T., Derakhshifar, I., Koglberger, H., Nowotny, N., 2006. Occurrence of six honeybee viruses in diseased Austrian apiaries. Appl. Environ. Microbiol. 72 (4), 2414–2420.

Beuve, M., Sempe, L., Lemaire, O., 2007. A sensitive one-step real-time RT-PCR method for detecting Grapevine leafroll-associated virus 2 variants in grapevine. J. Virol. Methods 141 (2), 117–124.

Blanchard, P., Ribiere, M., Celle, O., Lallemand, P., Schurr, F., Olivier, V., Iscache, A.L., Faucon, J.P., 2007. 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. J. Virol. Methods 141 (1), 7–13.

Bowen-Walker, 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 (1), 101–106.

Chen, Y.P., Zhao, Y., Hammond, J., Hsu, H.T., Evans, J., Feldlaufer, M., 2004. Multiple virus infections in the honey bee and genome divergence of honey bee viruses. J. Invertebr. Pathol. 87 (2/3), 84–93.

Chen, Y.P., Higgins, J.A., Feldlaufer, M.F., 2005. Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.). Appl. Environ. Microbiol. 71 (1), 436–441.

Evans, J.D., Hung, A.C.F., 2000. Molecular phylogenetics and the classification of honey bee viruses. Arch. Virol. 145 (10), 2015–2026.

Genersch, E., 2005. Development of a rapid and sensitive RT-PCR method for the detection of deformed wing virus, a pathogen of the honeybee (*Apis mellifera*). Vet. J. 169 (1), 121–123.

Grabenstiner, E., Ritter, M.J., Carter, S., Davison, H., Pechhacker, J., Kolodziejek, O., Boeching, I., Derakhshifar, R., Moosbeckhofer, E., Licek, E., Nowotny, N., 2001. Sacbrood virus of the honeybee (*Apis mellifera*): rapid identification and phylogenetic analysis using reverse transcription-PCR. Clin. Diagn. Lab. Immunol. 8, 93–104.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986–994.

Hung, A.C.F., Shimanuki, H., 1999. A scientific note on the detection of Kashmir bee virus in individual honeybees and *Varroa jacobsoni* mites. Apidologie 30, 353–354.

Leat, N., Ball, B., Govan, V., Davison, S., 2000. Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. J. Gen. Virol. 81 (Pt. 8), 2111–2119.

Martin, S.J., 2001. *Varroa destructor* reproduction during the winter in *Apis mellifera* colonies in UK. Exp. Appl. Acarol. 25 (4), 321–325.

Mayo, M.A., 2002. Virus taxonomy—Houston 2002. Arch. Virol. 147, 1071–1076.

Morse, R.A., Calderon, N.W., 2000. The value of honey bee pollination in the United States. Bee Cult. 128, 1–15.

Nordstrom, S., 2003. Distribution of deformed wing virus within honey bee (*Apis mellifera*) brood cells infested with the ectoparasitic mite *Varroa destructor*. Exp. Appl. Acarol. 29 (3/4), 293–302.

Ribière, M., Triboulot, C., Mathieu, L., Aurières, C., Faucon, J.P., Pépin, M., 2002. Molecular diagnostic of chronic bee paralysis virus infection. Apidologie 33, 339–351.

Richardson, J., Molina-Cruz, A., Salazar, M.A., Black, V., 2006. Quantitative analysis of Dengue-2 virus RNA during the extrinsic incubation period in individual *Aedes aegypti*. Am. J. Trop. Med. Hyg. 74 (1), 132–214.

Shimanuki, H., Calderone, N.W., Knox, D.A., 1994. Parasitic mite syndrome: the symptoms. Am. Bee J. 134 (12), 827–828.

Stoltz, D., Shen, X., Boggis, R., Sisson, C.G., 1995. Molecular diagnosis of Kashmir bee virus infection. J. Apic. Res. 34, 153–165.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M.E., Bergoin, M., 2004. Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. Appl. Environ. Microbiol. 70 (12), 7185–7191.

Topley, E., Davison, S., Leat, N., Benjeddou, M., 2005. Detection of three honeybee viruses simultaneously by a single multiplex reverse transcriptase PCR. Afr. J. Biotechnol. 4, 763–767.