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Simultaneous detection of bee viruses by multiplex PCR

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A B S T R A C T

Honey bee mortality is a serious problem that beekeepers in Argentina have had to face during the last 3 years. It is known that the consequence of the complex interactions between environmental and beekeeping parameters added to the effect of different disease agents such as viruses, bacteria, fungi and parasitic mites may result in a sudden collapse of the colony. In addition, multiple viral infections are detected frequently concomitantly in bee colonies. The aim of this study was to establish a multiplex polymerase chain reaction method for rapid and simultaneous detection of the most prevalent bee viruses. This multiplex PCR assay will provide specific, rapid and reliable results and allow for the cost effective detection of a particular virus as well as multiple virus infections in a single reaction tube. This method could be a helpful tool in the surveillance of the most frequently found bee viruses and to study the dynamics and the interactions of the virus populations within colonies.

1. Introduction

Honeybees (*Apis mellifera*) are the most important insect pollinators which are crucial for the maintenance of global agricultural production (Morse and Calderone, 2000). Recently, honeybee population experienced losses worldwide due to unknown causes. Several pathogens have been proposed to explain these losses. Some viruses appear to be the most probable cause of bee's death, such as Deformed wing virus (DWV) (Martin et al., 2012) or Acute bee paralysis virus (ABPV) (Bailey et al., 1963; Govan et al., 2000; Genersch, 2010). Although Israeli acute paralysis virus (IAPV) was first describe as a predictive marker of colony losses in USA (Cox-Foster et al., 2007), subsequent surveys indicate that this virus was not the main factor responsible for losses but only one of multiple possible factors involved (vanEngelsdorp et al., 2009; de Miranda et al., 2010). In this way, others authors suppose that a possible cause of the unexplained bee losses could be combinations of pathogens such as an Invertebrate iridescent virus (IIV) with *Nosema ceranae* (Bromenshenk et al., 2010) or DWV with *Varroa destructor* (Dainat et al., 2011).

Multiple viral infections are detected frequently in bee colonies (Chen et al., 2004; Ellis and Munn, 2005; Tentcheva et al., 2004). At least, 18 honeybee viruses have been isolated, characterized and described in the literature (Allen and Ball, 1996; Genersch, 2010), and more recently four new viruses were suggested as a possible cause of infection in honeybees (Runckel et al., 2011). In general, these viruses are 30-nm isometric particles containing a single-stranded positive RNA. Even though the abundance of honeybee virus is different between continents worldwide, some of the most intensively studied and common viruses are: IAPV, Kashmir bee virus (KBV) and Acute bee paralysis virus (ABPV) are three species of the genus *Aparavirus*, while Black queen cell virus (BQCV) is specie of the genus *Cripavirus*, and both genus belong to the family *Dicistroviridae*; DWV and Sacbrood bee virus (SBV) are species from the genus *Iflavirus* of the family *Iflaviridae.* Finally, Chronic bee paralysis virus (CBPV) is still unclassified (ICTV, 2012). All those viruses are widely distributed worldwide (Allen and Ball, 1996; Ellis and Munn, 2005; Reynaldi et al., 2010, 2011).

ABPV was originally considered an economically irrelevant viral pathogen of honeybees, because of its lack of clinical signs. Subsequently, was shown to be responsible for both brood and adult bee mortality in colonies infested with *Varroa destructor* (Bekesi et al., 1999; Hung et al., 1995). In contrast, other viruses that produce clinical signs can be clearly identifiable by beekeepers (Bailey and Ball, 1991). BQCV causes mortality especially in queen prepupae and pupae, which is considered to be transmitted by *Nosema apis* infestation (Allen and Ball, 1996). CBPV causes a characteristic trembling, flightless, and sometimes bees turn black, hairless, shiny and crawling at the entrance of the hive. SBV affected larvae change from pearly white to gray and finally black, the head region

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Table 1
Designed primers, nucleotide sequences and sizes of amplicons for each viral species.

Primer	Virus	Nucleotide sequences (5′–3′)	GenBank (accession number)	Genomic position	Product length (bp)
AIVf IAPVr	IAPV	GGTGCCCTATTTAGGGTGAGGA GGGAGTATTGCTTTCTTGTTGTG	HQ897161	6490–6511 6647–6625	158
DWVf DWVr	DWV	TGGTCAATTACAAGCTACTTGG TAGTTGGACCAGTAGCACTCAT	GU109335	2170–2191 2438–2417	269
SBVf SBVr	SBV	CGTAATTGCGGAGTGGAAAGATT AGATTCCTTCGAGGGTACCTCATC	AF092924	314–336 655–632	342
AIVf ABPVr	ABPV	GGTGCCCTATTTAGGGTGAGGA ACTACAGAAGGCAATGTCCAAGA	AF486073	6398–6419 6857–6835	460
BQCVf BQCVr	BQCV	CTTTATCGAGGAGGAGTTCGAGT GCAATAGATAAAGTGAGCCCTCC	EF517520	7762–7784 8297–8275	536
CBPVf CBPVr	CBPV	AACCTGCCTCAACACAGGCAAC ACATCTCTTCTTCGGTGTCAGCC	EU122229	1802–1823 2575–2553	774

is usually darker than the rest of the body that appears to be a sack filled with water (Allen and Ball, 1996). Bees infected with IAPV present shivering wings, progressing to paralysis, and die outside the hive (Maori et al., 2007). DWV produce wing deformity and other visible morphological abnormalities in workers and drones (Bowen-Walker et al., 1999; Yue and Genersch, 2005). KBV have similar symptoms as ABPV. In honey bee population, KBV is prevalent in North America and New Zealand (Bruce et al., 1995; Todd et al., 2007) but rarely found in Europe (Siede et al., 2005), and has not been reported in South America. For this reason KBV was not included in this study.

Several methods have been developed for the detection of honeybee viruses such as agar gel immunodiffusion test (AGID), Enzyme-linked immunosorbent assay (ELISA), electron microscopy (EM) and Western blotting (WB). The AGID test has frequently been used for the diagnosis of honeybee viruses, because it is fast and inexpensive (Allen and Ball, 1996), but only certain laboratories have access to reliable antisera. ELISA is a much more sensitive means of virus detection and has the advantage of providing a quantitative estimate of virus present in a sample, when used in conjunction with virus standards of known concentration (Ball, 1999). Although, AGID and ELISA have less sensitivity when compared with molecular techniques. Electron microscopy and WB are useful tools in research laboratories but are expensive and time consuming techniques for routine diagnostic submissions. EM has low specificity, because most of bee viruses are similar in shape and size, so it is not possible to distinguish between them. Recently two new techniques have been developed, a multiplex-ligation probe dependent amplification (MLPA) called BeeDoc – MLPA for the detection of 10 honey bee virus (De Smet et al., 2012) and a microarray based technique capable of detecting viruses, microbes, and metazoans associated to arthropod (Runckel et al., 2011).

The reverse transcription polymerase chain reaction (RT-PCR) is a powerful technique, sensitive and highly specific and has been used to detect several bee viruses (Tentcheva et al., 2004; Benjeddou et al., 2001). Simplex PCR detects one target per reaction therefore different PCRs are required to detect multiple targets. The multiplex RT-PCR (RT-mPCR) is a useful technique for the simultaneous detection of different viruses in a sample, offering a significant time and cost-saving advantage, especially when large numbers of samples are analyzed. This technique has been used to detect viruses of olive tree (Bertolini et al., 2001), viruses of citrus trees (Roy et al., 2005) or viruses of tomato (Panno et al., 2012). Topley et al. (2005) and Grabensteiner et al. (2007) detected ABPV, BQCV and SBV honeybee viruses by RT-mPCR, while Meeus et al. (2010) could detect ABPV, KBV, IAPV and DWV from bumblebees in a single reaction. In this work, we describe the development and evaluation of a RT-mPCR assay for the simultaneous detection and differentiation of six bee viruses.

2. Materials and methods

2.1. Samples

One hundred and seventy samples (each one consisting of a pool of adult worker honeybees) were collected from different Argentine apiaries, taken from the main honey producing regions (Province of Buenos Aires, Córdoba, Santa Fe and Entre Rios). No symptom of bee disease such as American Foulbrood (*Paenibacillus larvae*) or varroatosis (*Varroa destructor*) was seen in these apiaries. The survey was carried out from December 2010 to March 2012 and the samples were stored at −70 °C until being processed.

2.2. Primer design

Nucleotide sequences of IAPV, DWV, SBV, ABPV, BQCV, KBV and CBPV (at least twenty different strains of each virus from diverse regions worldwide) obtained from GenBank, were aligned using the program CLUSTAL-X (http://www.clustal.org/clustal2/) and examined to search for conserved regions. Subsequently, different specific primers with similar annealing temperatures were designed. Internal structures as hairpins, self and heterodimers were avoided using the AnnHyb v4.944 program (http://bioinformatics.org/annhyb). To check primers specificity, local alignment was performed using the Primer-Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The oligonucleotide primers were custom-synthesized by Integrated DNA Technology (IDT, Coralville, IA, USA): **AIVf**: 5′-GGTGCCCTATTTAGGGTGAGGA-3′, corresponding to a conserved area within the non-coding intergenic region of *Aparavirus* (generic primer of both ABPV and IAPV); **ABPVr**: 5′-ACTACAGAAGGCAATGTCCAAGA-3′, corresponding to a specific capsid polyprotein sequence of ABPV; **IAPVr**: 5′-GGGAGTATTGCTTTCTTGTTGTG-3′ corresponding to a IAPV structural polyprotein; **DWVf**: 5′-TGGTCAATTACAAGCTACTTGG-3′ and **DWVr**: 5′-TAGTTGGACCAGTAGCACTCAT-3′ corresponding to conserved sequences of capsid proteins (VP2-VP1) of DWV; **SBVf**: 5′-CGTAATTGCGGAGTGGAAAGATT-3′ and **SBVr**: 5′-AGATTCCTTCGAGGGTACCTCATC-3′ corresponding to a conserved region of polyprotein of SBV; **CBPVf**: 5′-AACCTGCCTCAACACAGGCAAC-3′ and **CBPVr**: 5′-ACATCTCTTCTTCGGTGTCAGCC-3′, corresponding to RNA-dependent RNA polymerase conserved region of CBPV; **BQCVf**: 5′-CTTTATCGAGGAGGAGTTCGAGT-3′ and **BQCVr**: 5′-GCAATAGATAAAGTGAGCCCTCC-3′ corresponding to a partial capsid polyprotein gene of BQCV. The

orientation, genomic position, GenBank reference target and product length of primers used in mPCR is listed in Table 1.

2.3. Nucleic acid extraction

Three extraction methods were evaluated to select a reliable procedure to obtain the viral RNA: TRIzol reagent (Invitrogen, Carlsbad, CA, USA), SV Total RNA Isolation System (Promega, Madison, WI, USA) and Viral Nucleic Acid Extraction Kit II (Real Biotech, Taipei, Taiwan).

Initially fifteen bees, randomly selected from each sample, were crushed in stomacher bags with 2 ml of phosphate-buffered saline (PBS). After homogenization, the samples were centrifuged for 15 min at 500 × *g* to clarify. A second clarification was carried out at 5000 × *g* during 30 min. Five hundred microliters of the final supernatant was employed for total RNA extraction using each of the three above mentioned methods according to the manufacturer's protocol. In all cases, the RNA was resuspended in a final volume of 50 μl of nuclease-free water. Total RNA yield was calculated spectrometricaly based on absorbance at 260 nm. RNA quality was estimated by using the OD 260/280 absorbance ratio.

Finally, 5 μl of total RNA (obtained by each method) were used for synthesis of complementary DNA (cDNA). Reactions were carried out using the enzyme Moloney Murine Leukemia Virus (M-MLV; Promega) under conditions specified by the supplier, employing 40 ng of a mixture of random hexamer primers.

2.4. Simplex PCR

To confirm primer functionality, each primer pair was initially tested separately in a conventional PCR reaction. Positive samples identified in our laboratory in previous works (Reynaldi et al., 2010, 2011) were used as template.

Five microliters of cDNA were used in a 50 μl reaction volume containing 5 U of Taq DNA polymerase (Promega), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Promega) and 1 μM each of sense and antisense primers. To determine the best annealing temperature for a correct amplification preventing primer dimer formation, a gradient PCR was performed using the following parameters: one cycle at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 52–62 °C (with an increase of 2 °C per sample) for 30 s, and 72 °C for 45 s, followed by one cycle at 72 °C for 10 min. PCR products were analyzed by electrophoresis on 2.5% agarose. The bands were visualized by ultraviolet transillumination after staining with ethidium bromide at a final concentration of 0.5 μg/ml.

2.5. Multiplex PCR

Multiplex PCR amplification was optimized in a 25 μl reaction consisting of 0.5 μM of each of the oligonucleotide primers, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U of Taq DNA polymerase (Promega), 2.5 μl of 10× reaction buffer (supplied with the enzyme) and 5 μl of cDNA (0.5 μg approximately).

Annealing temperature for a correct amplification of all six viruses was determined by gradient PCR using the following parameters: one cycle at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 52–62 °C (with an increase of 2 °C per sample) for 30 s, and 72 °C for 45 s and a final extension cycle at 72 °C for 10 min. PCR products were analyzed by electrophoresis on a 2.5% agarose gel.

After electrophoresis, gel products were purified using a purification kit (Wizard SV Gel and PCR clean-up System; Promega) and sequenced using virus-specific primer pairs in both orientations (Biotechnology Resource Center, University of Cornell, Ithaca, USA). The obtained sequences were compared with homologous sequences from the GenBank to confirm the identity of each virus.

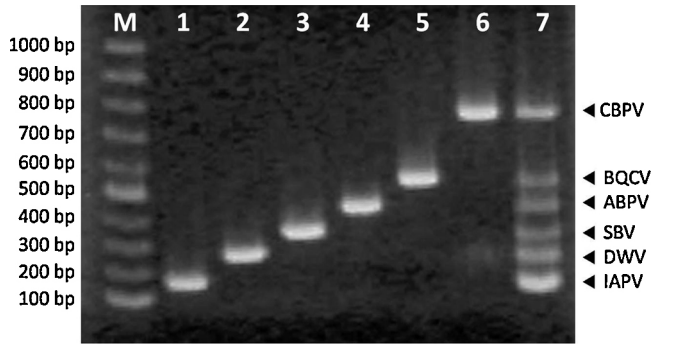

Fig. 1. Amplification of six bee virus by simplex PCR performed at 56 °C (lanes 1–6) and multiplex PCR lane 7. Lane 1: IAPV (158 bp); lane 2: DWV (269 bp); lane 3: SBV (342 bp); lane 4: ABPV (460 bp); lane 5: BQCV (536 bp); lane 6: CBPV (774 bp); M: 100 bp molecular marker (Fermentas).

For testing the sensitivity of mPCR, the total cDNA preparations from each virus sample were adjusted to a final concentration of 100 ng/μl and combined on identical proportion in a single tube. To compare the sensitivity of the method, 10-fold serial dilutions (10^{-1} to 10^{-6}) of a mixture of the six viral cDNA were made and employed as template for mPCR.

Once found the most suitable conditions for RNA extraction and mPCR reaction, we analyzed 170 bee samples from the main honey producing regions of Argentina. All samples were processed by mRT-PCR and checked with simplex PCR using primers already designed: IAPV (Reynaldi et al., 2011); ABPV, CBPV and SBV (Reynaldi et al., 2010); DWV (Tentcheva et al., 2004) and BQCV (Benjeddou et al., 2001).

3. Results

3.1. Nucleic acid extraction

All three methods resulted in acceptable RNA concentrations: 345.2 ± 54.3 ng/μl with TRIzol reagent, 527.3 ± 37.4 ng/μl with SV Total RNA Isolation System and 604.5 ± 41.7 ng/μl with Viral Nucleic Acid Extraction Kit II. No substantial differences were observed between the three extractions methods compared when ANOVA test was made ($p < 0.05$). Even though, the best rate of recovery and the highest purity (measured as the relation of absorbance at 260 and 280 nm) was obtained when Viral Nucleic Acid Extraction Kit II (Real Biotech) was used, therefore this method was preferred to process all samples collected in the field.

3.2. Simplex and multiplex PCR

All primers performed well in the tested temperature range (52–62 °C). However, we detected a notorious lack of amplification in ABPV controls at 60 °C and 62 °C both in simplex and in multiplex PCR (data not shown). In order to save this problem we established the optimal annealing temperature at 56 °C (Fig. 1). The developed mPCR detected viral nucleic acid up to a 10^5-fold dilution of the cDNA extracts of infected honey bees.

3.3. Processing of field-collected samples

No differences were observed between the mRT-PCR described in this work and other simplex PCRs previously reported. Even more, we did not find differences between simplex or multiplex PCR in the 170 samples analyzed by both methods. We have found a frequency of 45.9% for IAPV, 37.1% for DWV, 12.3% for CBPV, 13, 6% for SBV, 3.5% for BQCV and 7.7% for ABPV. Even more, this mPCR showed to be adequate for simultaneous detection of several

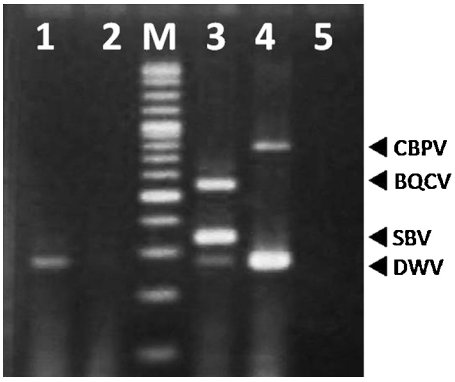

Fig. 2. Simultaneous detection of multiple bee viruses from field collected samples by mPCR. Lane 1: detection of a single infection by DWV (269 bp); lanes 2 and 5 negative samples for all searched viruses; M: 100 bp DNA ladder (genbiotech); lane 3: detection of a mixed infection by BQCV (536 bp), SBV (342 bp) and DWV (269 bp); lane 4: detection of a co-infection by CBPV (774 bp) and DWV (269 bp).

viruses in a single sample (Fig. 2). Co-infection with 4 viruses (ABPV, SBV, IAPV and DWV) was found in only one sample; 3 samples were positive for 3 viruses (two of them with SBV, DWV and IAPV and the other one with BQCV, SBV and DWV). Finally, 29 samples were found positive to different combinations of two viruses; most of them (17 samples out of 29) have the association of IAPV and DWV.

4. Discussion

The integrity of purified RNA is critical to all molecular techniques. The starting RNA sample is particularly important. The three methods analyzed for nucleic acid extraction resulted equally well and could serve to carry out the extraction of viral RNA.

The mPCR specifically detected the six target viruses. Sequencing of the amplified products showed nucleotide identities ranking from 98 to 100% (data not shown). The assay sensitivity was high, viral nucleic acid could be detected up to 10^5-fold cDNA dilution. Similar findings were reported from other authors (Grabensteiner et al., 2007; Roy et al., 2005). Theoretically, our detection level could identify even one single infected bee within a pool of several hundred, showing that the method could be useful in the detection of low levels virus infection.

The primers used in this study avoided the potential cross-amplification related to the ABPV-KBV-IAPV virus complex. Primers were designed based on a pattern of sequence conservation and variation within and between these virus groups. Other investigators reported problems with detection of viruses belonging to Genus Aparavirus, particularly ABPV, IAPV and KBV (not included in this work) because of their high homology (de Miranda et al., 2010). To the best of our knowledge, this is the first report of DWV and BQCV in Argentina.

Several mPCR methods were previously described for detection of bee virus; ABPV, SBV and BQCV (Topley et al., 2005; Grabensteiner et al., 2007), or in bumblebees; ABPV, DWV and KBV (Meeus et al., 2010). Recently, Carletto et al. (2010) have developed a method that could detect fourteen bee pathogens (seven of them are virus) but using four mPCRs. In contrast, the mPCR described here can detect six viruses simultaneously in a single reaction.

Other methods capable of detecting several viruses such as the MPLA (De Smet et al., 2012) and microarrays (Glober et al., 2011; Runckel et al., 2011), require more sophisticated and expensive equipment, which is often not available in some laboratories. Developing MLPA probe mixes is complicated, expensive and time-consuming; even more MLPA reactions are more sensitive to contaminants than ordinary PCR reactions. In contrast, the mPCR is more reliable, rapid and cost-effective diagnostic for detection of multiple bee viruses in a single sample. It might be useful also for detecting unapparent, subclinical viral infection in apiaries which contribute to the spread of these viruses from one hive to another. Accurate identification of infected bees and asymptomatic carriers is essential for the understanding of the viral epidemiology in apiaries. This method could be a helpful tool in the surveillance of the most commonly found bee viruses and to understand the dynamics and the interactions of the virus populations within colonies.

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