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Journal of Invertebrate Pathology 90 (2005) 69–72
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Short communication

Detection of Chronic bee paralysis virus and Acute bee paralysis virus in Uruguayan honeybees

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Received 6 April 2005; accepted 19 July 2005
Available online 15 September 2005

Abstract

Chronic bee paralysis virus (CBPV) causes a disease characterized by trembling, flightless, and crawling bees, while Acute bee paralysis virus (ABPV) is commonly detected in apparently healthy colonies, usually associated to *Varroa destructor*. Both viruses had been detected in most regions of the world, except in South America. In this work, we detected CBPV and ABPV in samples of Uruguayan honeybees by RT-PCR. The detection of both viruses in different provinces and the fact that most of the analyzed samples were infected, suggest that, they are widely spread in the region. This is the first record of the presence of CBPV and ABPV in Uruguay and South America.

Keywords: Honeybees; *Apis mellifera*; Chronic bee paralysis virus; Acute bee paralysis virus; Honeybee mortality; RT-PCR

In Uruguay, South America, a country that has a population of about 3 million people, there are about 4000 beekeepers, more than 400,000 beehives and during the last years honey has become one of the most important agricultural products for export.

However, mortality of honeybees is one of the most serious problems that beekeepers have to face periodically. The causes are not well understood. Probably, there are several factors involved, like the presence of *Varroa destructor* and *Paenibacillus larvae* subsp. *larvae*, etiological agents of Varroosis and American foulbrood, respectively and intoxication with insecticides or pesticides used in agriculture (Suchail et al., 2004). It has also been suggested that episodes of mortality worldwide are related to the presence of RNA viruses. More than 18 viruses that affect honeybees have been described, including Chronic bee paralysis virus (CBPV) and Acute bee paralysis virus (ABPV) (Ball and Bailey, 1991).

CBPV causes an infectious disease characterized by trembling, flightless and sometimes black individuals crawling at the hive entrance (Allen and Ball, 1996).

ABPV is a common infective agent of bees, frequently detected in apparently healthy colonies (Allen and Ball, 1996). However, it has been reported as the major factor contributing to the mortality of honeybees affected with *V. destructor* (Faucon et al., 1992; Nordstrom et al., 1999).

The presence of both viruses has been reported worldwide, although so far there are no records about their presence in South America (Allen and Ball, 1996).

One of the most accurate methods for the diagnosis of RNA viruses is reverse transcription-polymerase chain reaction (RT-PCR). This technique provides a quick, specific, and sensitive procedure for the diagnosis of virus in samples. RT-PCR has been previously used for the detection of several honeybee viruses (Benjeddou et al., 2001; Ribiere et al., 2002). Ribiere et al. (2002) designed specific primers for the detection of CBPV that

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0022-2011/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2005.07.001

amplify a 455 bp fragment of the sequence of the putative viral CBPV RNA polymerase gene. Benjeddou et al. (2001) designed specific primers for the detection of ABPV. In this case, these primers amplify a 900 bp fragment that corresponds to the protein of the viral capsid.

In the present work, we used the primers designed by these authors to detect CBPV and ABPV in adult honeybees from different geographic regions of Uruguay.

Thirty-six worker honeybee (*Apis mellifera*) samples from different provinces of Uruguay [Colonia and Soriano (west), Canelones and San José (south), Maldonado and Lavalleja (east), and Rivera (north)] were used in this study. Honeybees were collected from December 2003 to December 2004, and most of them were associated to mortality episodes that periodically occur, mainly in winter, in recent years.

Samples were sent refrigerated to the Laboratory of Microbiology, IIBCE (Montevideo, Uruguay), and processed immediately after being received. The sources and symptoms corresponding to each sample are shown in Table 1.

Ten honeybees were randomly selected from each sample and placed in sterile plastic bags using sterile forceps and 10 ml of phosphate-buffered saline (PBS) was added. Bees were crushed for 2 min at high speed in a Stomacher 80 Lab Blender (Seward, London, UK) and the resultant homogenate was first centrifuged at 1500*g* for 10 min. The supernatant was recovered and centrifuged again at 12,000*g* for 15 min and 140 μl of the final supernatant was used for viral RNA extraction.

RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of RNA and DNA amplification were performed using a continuous RT-PCR method One-Step RT-PCR kit (Qiagen), according to the manufacturer's recommendations. PCR was performed in a final volume of 50 μl including 10 μl of 5× buffer, 2 μl of dNTP Mix (containing 10 mM of each dNTP), 1.5 μl of a stock solution of 20 μM of each primer, 2 μl of Enzyme Mix, and 2 μl of template RNA. Negative RT-PCR controls were carried out excluding nucleic acids from the reaction. An

Table 1
Analysis of the presence of CBPV and ABPV in honeybees from Uruguay, by RT-PCR

Sample	Date	Province	Predominant symptom	CBPV	ABPV
V5	December-2003	San José	Honeybees mortality	+	–
V6	February-2004	San José	Healthy honeybees	–	–
V7	March-2004	Colonia	No data	+	–
AL	March-2004	Colonia	No data	+	+
3L	March-2004	Colonia	No data	–	+
V9	May-2004	Colonia	No data	–	+
V10	May-2004	Colonia	No data	+	–
V12	June-2004	Colonia	Deformed wings	–	+
V13	June-2004	Colonia	Healthy honeybees	+	+
V14	June-2004	Colonia	Healthy honeybees	+	+
V15	June-2004	Colonia	Healthy honeybees	+	+
V16	June-2004	Colonia	Honeybees mortality	+	+
V17	June-2004	Colonia	Honeybees mortality	–	+
V18	June-2004	Maldonado	Deformed wings	+	+
V19	June-2004	Maldonado	Honeybees mortality	+	+
V20	June-2004	Maldonado	Healthy honeybees	+	+
V21	June-2004	Maldonado	Honeybees mortality	+	–
V22	May-2004	Rivera	Honeybees mortality	+	–
V23	June-2004	Lavalleja	Healthy honeybees	–	–
V24	June-2004	Lavalleja	Healthy honeybees	+	–
V25	June-2004	Lavalleja	Honeybees mortality	+	+
V26	June-2004	Lavalleja	Honeybees mortality	+	–
V27	June-2004	Lavalleja	Deformed wings	+	+
V28	June-2004	Colonia	No data	+	–
V29	July-2004	San José	No data	+	+
V30	July-2004	Rivera	No data	+	+
V31	July-2004	Colonia	No data	+	–
V32	August-2004	Canelones	Honeybees mortality	–	–
V33	August-2004	Canelones	Honeybees mortality	–	+
V40	July-2004	Soriano	No data	–	–
V46	September-2004	Maldonado	No data	–	–
V50	September-2004	Colonia	No data	–	–
V52	September-2004	Maldonado	Honeybees mortality	+	–
V57	October-2004	Maldonado	Honeybees mortality	–	–
V58	November-2004	Colonia	No data	–	–
V59	December-2004	Canelones	Honeybees mortality	–	+

additional control was performed adding RNA from healthy honeybees. The RT-PCR program included a reverse transcription stage at 50 °C for 30 min, followed by an initial PCR activation step at 95 °C for 15 min. This was followed by 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The reaction was performed using a T1 Biometra Thermocycler and products were visualized by electrophoresis in 0.8 % (w/v) agarose gels stained with ethidium bromide (Sambrook et al., 1989).

Ten samples gave a unique band of 450 bp, corresponding to CBPV, six samples gave a unique band of 900 bp corresponding to ABPV, twelve samples presented both bands, indicating that those colonies were co-infected by both virus and only eight samples resulted negative. The results of RT-PCR are shown in Fig. 1 and Table 1.

Thirteen samples were from apiaries in which bee mortality episodes occurred and most of them were infected with CBPV or ABPV or even with both viruses simultaneously. Honeybees of three samples exhibited deformed wings (a symptom related to the deformed wings virus) and were infected with both viruses. Lastly, seven samples included apparently healthy honeybees, but five of these were infected, confirming the presence of viruses in an unapparent state. Both negative controls (without nucleic acids and including RNA from healthy bees) did not show any band. The detection of both viruses in honeybees from different and distant locations, and the fact that most of the samples (78%) were infected with one or both viruses, suggest that they are widely spread in the country.

One 455 bp DNA fragment amplified with CBPV primers (sample V5) and another 900 bp DNA fragment amplified with ABPV primers (sample AL) were excised from the agarose gel, purified using the Concert Rapid Gel Extraction System kit (Life Technologies) and cloned into a pGEM-T Easy Vector System (Promega) according to manufacturer's instructions. DNA sequencing of the cloned inserts that corresponded to the selected PCR products was performed at the Faculty of Sciences (University of Uruguay), using an Applied Biosystems automated DNA sequencer (model 377). The nucleotide sequences were submitted to the GenBank database of the National Center for Biotechnology Information (NCBI, Bethesda, USA) (Accession Nos. AY763287 for CBPV and AY763414 for ABPV).

A BLAST search of the CBPV amplification product sequence, using the GenBank Nucleotide database, yielded a high percentage of similarity (87%) with published CBPV sequences. The nucleotide sequence was then translated and compared again with the GenBank Protein database, obtaining a 98% similarity.

The BLAST search of the ABPV amplicon sequence gave a high percentage of similarity (84%) with the corresponding ABPV published sequences. The translated nucleotide sequence was compared to the GenBank Protein database and similarity was 85%.

The high percentage of identity between nucleotide or predicted amino acid sequences of both virus and previously published sequences, confirmed their identification. The genomic diversity observed between native and published sequences of very distant geographic areas is a convenient indicator for the identification and classification of isolates of different origins (Bakonyi et al., 2002).

In summary, this is the first record of the presence of CBPV and ABPV in Uruguay and South America. The high rate of infection, simultaneous co-infection of several colonies by both viruses and the viral presence in healthy bees suggest that CBPV and ABPV are probably widely spread in the region.

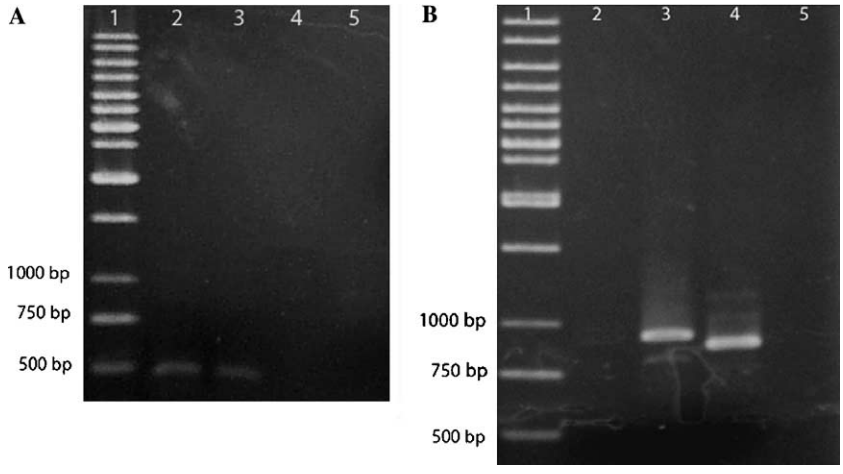

Fig. 1. RT-PCR results using specific primers for the detection of CBPV and AVPB. (A) Detection of CBPV. 1, Generuler 1 kb DNA ladder (Fermentas, Canada); 2, sample V5; 3, sample AL; 4, negative control without RNA; 5, negative control using RNA from healthy bees. (B) Detection of ABPV 1, Generuler 1 kb DNA ladder (Fermentas, Canada); 2, negative control without RNA; 3, sample V7; 4, sample AL; 5, negative control using RNA from healthy bees.

Acknowledgments

This work was supported by the Instituto Nacional de Investigación Agropecuaria (INIA, Uruguay). The authors thank Mercedes Peyrou for discussion of technical aspects of this work.

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