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First report of Israeli acute paralysis virus in asymptomatic hives of Argentina

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ABSTRACT

Honey bee mortality has recently been associated with Israeli acute paralysis virus (IAPV), a proposed etiological agent for a new syndrome known as Colony Collapse Disorder. Bees infected with this virus show shivering wings, progress into paralysis, and finally die outside the hive. During the last years, honey bee mortality became a serious problem for Argentinean beekeepers. We herein report the preliminary results of a survey carried out to detect IAPV in samples taken from several Argentine provinces, by using a reverse transcription Polymerase Chain Reaction assay. Our data indicate the existence of high frequency of IAPV in asymptomatic hives of Argentina.

Key words: honey bee, Israeli acute paralysis virus, RT-PCR

RESUMEN

Primera comunciación del virus israelí de la parálisis aguda en colmenas asintomáticas de la República Argentina. Recientemente la mortalidad de las abejas melíferas ha sido asociada al virus israelí de la parálisis aguda (IAPV), propuesto como agente etiológico del denominado síndrome de despoblamiento de las colmenas. Las abejas infectadas con este virus presentan temblores en las alas que progresan hasta convertirse en parálisis, y finalmente mueren fuera de la colmena. Durante los últimos años, la mortalidad de las abejas melíferas se ha transformado en un serio problema para los productores de miel de la Argentina. Nosotros informamos aquí los resultados preliminares de un estudio realizado para detectar IAPV en muestras de colmenas provenientes de varias provincias argentinas utilizando la técnica de transcripción reversa-reacción en cadena de la polimerasa. Nuestros datos indican la presencia de IAPV en un alto porcentaje de las colonias estudiadas.

Palabras claves: abejas melíferas, virus israelí de la parálisis aguda, RT-PCR

Honey is one of the most important agricultural export products in Argentina. In fact, more than 3.5 million beehives and 50,000 beekeepers are related to this production, which is mainly located in Buenos Aires province. The international exchange of honeybee colonies and bee products has aroused greater interest in infectious diseases of bees. In particular, multiple viral infections are frequently detected concomitantly in bee colonies (3) and generally in an inapparent form of presentation (6). So far, at least 18 different viruses which are able to infect honeybees have been identified. These viruses have worldwide distribution. Acute bee paralysis virus (ABPV), Chronic bee paralysis virus (CBPV) and Sacbrood virus (SBV) have recently been detected in Argentina (9). Lately, severe bee mortality has inflicted heavy losses on Israeli (7) and the USA (4) apiculture. Bees exhibited symptoms reminiscent of those inflicted by ABPV, therefore the isolated virus was tentatively named Israeli acute paralysis virus (IAPV). IAPV-infected bees presented shivering wings, progressed to paralysis, and died outside the hive. Nowadays, the presence of IAPV has been strongly correlated with a new syndrome of honey bee losses observed in the United States and France, known as the Colony Collapse Disorder (CCD) (4). IAPV was characterized as a new member of the *Dicistroviridae* family, closely related to the Kashmir bee virus (KBV) and ABPV, but genetically and serologically different (7).

In this global scenario, viral bee diseases (*Apis mellifera*) are major economic points of consideration in apiculture. Given the importance of honey bees as pollinators and the reported association between CCD and IAPV, the aim of this work was to determine the frequency of IAPV in asymptomatic bee hives of Argentina.

Due to difficulties with the classical diagnostic methods in bee virology (i.e., absence of bee tissue cultures,

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Table 1. Primers used for the detection of Israeli acute paralysis virus (IAPV)

Primer*	Sequence (5´-3´)	Length (bp)	Amplification target	GenBank Accession N°
IAPV 1	AGGTGCCCTATTTAGGGTGAGGA	185	6598-6620	EU218534
IAPV 2	CGAAGCGAGTTCCGTATTGTGTAC		6783-6760	EU218534

*KBV and ABPV are not amplified with these primers.

seasonal dependence to obtain pupae for experimental infection, or requirement of type-specific serum for agar gel immunodiffusion tests), scientific interest has turned towards molecular techniques. Since complete or at least partial genome sequence information on the aforementioned viruses is available in gene bank databases, diagnostic methods based on reverse-transcription PCR (RT-PCR) have been developed for the detection of viral RNA in honeybee samples (3).

One hundred and forty one samples (consisting of a pool of 30-50 adult worker honeybees) from the most important honey producer provinces of Argentina (Buenos Aires, Córdoba, Santa Fe, Entre Ríos, San Luis and Río Negro) were used in this study. All samples were taken from apiaries without clinical symptoms of bee diseases, particularly American Foulbrood (*Paenibacillus larvae*) and varroatosis (*Varroa destructor*). The survey was carried out from March 2009 to March 2010 and samples were processed according to Reynaldi *et al.* (9) with modifications. Briefly, fifteen bees randomly selected from each sample were crushed in a mortar with 2 ml of phosphate-buffered saline (PBS). After homogenization, the samples were centrifuged for 15 min at 1,500 *x* g to clarify and, immediately a second clarification for 15 min at 15,000 *x* g was carried out using 1,000 µl of the first supernatant. Total RNA was extracted using 500 µl of Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) and mixed with 500 µl of the supernatant. The mixture was extracted with 220 µl of chloroform. After centrifugation at 12,000 *x* g for 10 min, the RNA contained in the aqueous solution was precipitated by adding an equal volume of isopropanol. The precipitated RNA was collected by centrifugation at 12,000 *x* g for 10 min, washed by 70 % ethanol and dissolved in 50 µl of RNase-free water. Five microlitres (approximately 7 ng) measured by spectrophotometer (SmartSpec 3000, Bio-Rad, Hercules, CA, USA) of total RNA was used for synthesis of complementary DNA (cDNA). The reaction was carried out using Moloney´s Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Promega Corp, Madison, WI, USA), employing 40 ng of a mixture of random primers under conditions specified by the supplier. Five microlitres of cDNA were added to a reaction mixture containing 2.5 µl of 10x buffer [75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01 % Tween 20], 0.75 µl of 10 mM dNTP mixture (Takara Shuzo Co. Ltd., Otsu, Japan), 1.5 µl of $MgCl_2$, 0.5 µM of each primer, 13.75 µl of RNase-free water and 0.5 µl (2.5 U) of Taq DNA polymerase (Fermentas Inc., Glen Burnie, MD, EE.UU.). The PCR reaction was performed in a final volume of 25 µl. Negative PCR controls were prepared by excluding the cDNA from the reaction. The PCR was carried out using a thermal protocol consisting of one denaturalization cycle of 5 min at 95 °C, followed by 40 amplification cycles [30 s at 95 °C, 30 s at 53 °C and extension time of 25 s at 72 °C]. A final extension cycle of 5 min at 72 °C was added to complete the polymerization. For IAPV amplification, primers expected to generate an amplicon of 185 bp were designed (Table 1). The specificity of PCR products was checked by sequencing. The detection limits of the RT-PCR reaction were determined by ten-fold serial dilutions and the highest dilution at which RT-PCR showed positive results was 10^{-6}.

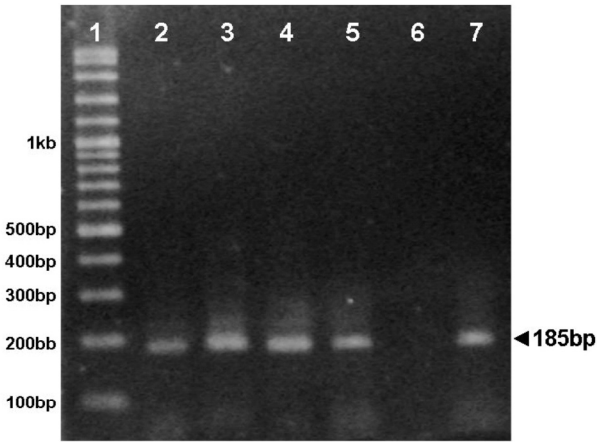

Figure 1. Electrophoresis of PCR products for bees with viral infections. Lane 1: 100 bp DNA ladder (Genbiotech). Lanes 2-5: Positive amplification for IAPV. Lane 6: Negative control (not template). Lane 7: Positive control.

IAPV was first described in Israel (7) and it was associated with bee mortality, but only a few hives in an apiary were affected. Even though the presence of IAPV in a hive was proposed to have high positive predictive value for CCD (4), the infection was described in the USA before the presence of CCD. Moreover, it was found in Spain (5) and France (2) in 18 % and 14 % of apiaries, respectively. Those apiaries had CCD symptoms, but in

none of both surveys a relationship between IAPV and CCD was found. In this study, the frequency of IAPV detection in Argentine honeybees from apiaries without clinical symptoms (including CCD) was 41 % (Figure 1). Although this frequency was high, other pathogens affecting the health status of the apiaries, such as *Paenibacillus larvae* (1) and *Varroa destructor* (Laboratorio de Sanidad Apícola, Ministerio de Asuntos Agrarios, unpublished data), are more prevalent in Argentina. Thus, IAPV does not seem to be a good marker for depopulation and colony loss, although the exact pathogenic role of this virus in Argentine honey bee colonies remains to be defined. In addition, these results suggest that genetic diversity among honey bees, other pathogens/factors, might explain the different susceptibility/resistance between hives in an apiary. To our knowledge, this is the first report of IAPV in South America. Future studies are needed to investigate the prevalence of IAPV in Argentina and to determine the existence of distinct IAPV lineages that are proposed to have implications for virulence (8**)**.

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