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First report of viral infections that affect argentine honeybees

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Summary

Honey is one of the most important agricultural products for export in Argentina. In fact, more than 3.5 million beehives and 50 000 beekeepers are related with this production, mainly located in Buenos Aires province. Honeybee 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 frequently detected concomitantly in bee colonies. We describe here the preliminary results of a survey of three honeybee-pathogenic viruses, acute bee paralysis viruses (ABPV), chronic bee paralysis viruses (CBPV) and Sacbrood viruses (SBV) detected during a screening of 61 apiaries located in the main honey producer province using a RT-PCR assay. This is the first molecular report of the presence of these viruses in Argentine apiaries.

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Introduction

Multiple viral infections are frequently detected concomitantly in bee colonies (Chen *et al*., 2004; Tentcheva *et al*., 2004; Ellis and Munn, 2005). Some of them may not cause characteristic clinical signs, as the case of the acute bee paralysis virus (ABPV) but, on the other hand, the chronic bee paralysis virus (CBPV) and the Sacbrood virus (SBV) produce clinical signs that can be clearly identifiable by beekeepers (Bailey and Ball, 1991). 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 grey and finally black, the head region is usually darker than the rest of the body that appears to be a sack filled with water.

In this worldwide scenario, and taking into account the existence of epidemiological records of bee loses related to viruses in different countries of South America such as in Brazil (Teixeira *et al*., 2008) and Uruguay (Antúnez *et al*., 2005; 2006), viruses have emerged as one of the candidates for this mortality in the region. However, as there are no reported data on these viruses in Argentina, the aim of the present work was to investigate the presence of ABPV, CBPV and SBV in apiaries of Buenos Aires province.

Sixty-one samples (consisting of a pool of 30–50 adult worker honeybees) from apiaries of different regions of Buenos Aires Province (cities of La Plata, Berisso, Ensenada, Magdalena, Florencio Varela, Brandsen, Chascomús, Vieytes, San Fernando, Lobos, Roque Pérez) were used in this study. All samples were taken from apiaries with a recent history of sudden collapse of colonies. No clinical sings of American Foulbrood (*Paenibacillus larvae*) or varroatosis (*Varroa destructor*) were seen in these apiaries. The survey was carried out from December 2008 to March 2009 and samples were stored at –70°C until being processed.

Fifteen bees randomly selected from each sample were crushed in a mortar with sterile sand and 2 ml of phosphate-buffered saline (PBS). After homogenization, the samples were centrifuged for 15 min at 1500 *g* to clarify and, immediately a second clarification was carried out using 1000 μl of the first supernatant. Total RNA was extracted using 500 μl of Trizol reagent Gibco BRL® and mixed with 500 μl of the supernatant. The mixture was

extracted with 220 μl of chloroform. After centrifugation at 12 000 *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 120 00 *g* for 10 min, washed by 70% ethanol and dissolved in 50 μl of RNase-free water. Five microlitres (approximately 7 μg) measured by spectrophotometer (SmartSpec 3000, Bio-Rad, Hercules, CA, USA) of total RNA was used for synthesis of complementary DNA (cDNA). This reaction was 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 Primers.

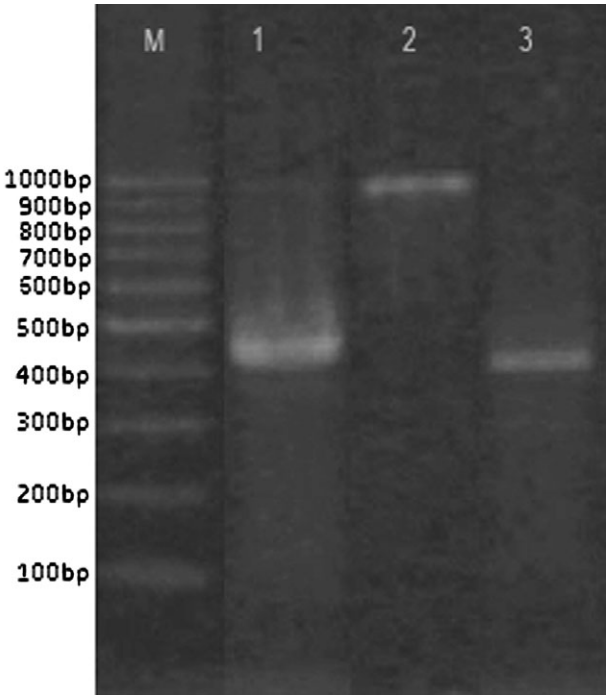

Fig. 1. Electrophoresis of PCR products for bees with viral infections. Line M: molecular weight marker (100–1500 bp). Positive amplification for CBPV (line 1), ABPV (line 2) and SBV (line 3) virus. The PCR reaction was performed in a final volume of 25 μl. Five microlitres of cDNA was added to a reaction mixture containing 2.5 μl of 10× 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 SHUZOCO®, 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®. 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 2 min at 95°C, followed by 40 amplifications cycles [30 s at 95°C, 30 s at the corresponding annealing temperature (53°C for SBV and CBPV or 59°C for ABPV), and extension time of 45 s for SBV and CBPV and 60 s for ABPV at 72°C]. To complete the polymerization a final extension single cycle of 5 min at 72°C was added. For the amplification of SBV and CBPV previously published primer pairs (Teixeira *et al*., 2008) that are expected to generate amplicons of 426 bp and 455 bp, respectively, were used. For ABPV another previously published primer pair (Benjeddou *et al*., 2001) that generates an amplicon of 900 bp was used.

Table 1. Comparative data of ABPV, CBPV and SBV in adult honeybee samples investigated in Brazil (Teixeira *et al*., 2008), Uruguay (Antúnez *et al*., 2006) and Argentina (this study).

Viruses	Argentina (%) 61 bee samples	Uruguay (%) 52 bee samples	Brazil (%) 192 bee samples
ABPV	16.3	9.0	27.1
CBPV	26.2	47.0	0
SBV	14.7	100[a]	0
Simultaneous infections			
0 virus	54.1	13.5	72.9
1 virus	39.5	76.9	27.1
2 viruses	6.4	9.6	0
3 viruses	0	0.0	0

a. Only 20 samples investigated.

The positive controls provided by Dr Karina Antúnez (Instituto de Investigaciones Biológicas Clemente Estable, Uruguay) were used in all PCR amplifications.

The amplification products were analysed by agarose gel electrophoresis and stained with ethidium bromide (Sambrook *et al*., 1989). The molecular size of the fragments was compared with that of a molecular weight marker (100–1500 bp).

To compare the sensitivity of PCR, 10-fold serial dilutions (10^{-1} to 10^{-6}) in nuclease-free water containing the total RNA extraction were tested.

The PCR products were purified using a gel extraction kit (Wizard® SV Gel & PCR Clean Up, Promega) and sequenced (Biotechnology Resource Center, University of Cornell, Ithaca, USA). The sequences were analysed using Basic Local Alignment Search Tool (BLAST) software.

Results and discussion

We describe here the first detection of three honeybee viruses (ABPV, CBPV and SBV) in Argentine apiaries (Fig. 1) by PCR. The detection limits of the RT-PCR reactions were determined and the highest dilution at which RT-PCR showed positive results was 10^{-6} for ABPV, 10^{-5} for SBV and CBPV. BLAST result confirmed the identity of the PCR-amplified sequences. Particularly, ABPV showed 98% of homology with AY763414 sequence, CBPV 100% of homology with AY763287 sequence and SBV 94% of homology with AF092924. The highest percentage of infection found in this study was for CBPV, which occurred in 16 (26.2%) of the 61 investigated apiaries. The second most frequently detected bee virus was ABPV, which was found in 10 (16.3%) of the 61 apiaries. Finally, SBV was detected in 9 (14.7%) apiaries. Only 4 apiaries (6.4%) were co-infected with at least two viruses: 2 (3.2%) with ABPV and SBV, and 2 (3.2%) with CBPV and SBV.

Even though we did not find simultaneous co-infections with ABPV, CBPV and SBV (Table 1), we might speculate that the relationship of these viruses added to the pres-

ence of several factors like other pathogens, environmental or beekeeping parameters might contribute to the sudden collapse of beehives in Argentine apiaries. To worsen this scenario, Alippi and colleagues (2007) reported the finding of isolates of *P. larvae* resistant to tetracycline, one of the two antibiotics approved to treat American Foulbrood in Argentina. Moreover, Maggi and colleagues (2009) reported *V. destructor* resistance to coumaphos, the most widely used acaricide in Argentina. On the other hand, the emerging pathogen *Nosema ceranae* which was found to be cause of colony collapse in bee hives in Spain (Higes *et al*., 2009) was recently reported in Argentine honeybees (Plischuk *et al*., 2008) and three species of Argentine native bumblebees (Plischuk *et al*., 2009).

In this preliminary report we found a low rate of infection and few cases of co-infection with more than one virus in the studied apiaries when compared with other countries of South America (Antúnez *et al*., 2006; Teixeira *et al*., 2008). Our result demonstrated that the viruses investigated in this survey were widespread in the analysed Buenos Aires apiaries.

In summary, this study describes the first molecular report of the presence of ABPV, CBPV and SBV in Argentina. The results added to the presence of characteristic clinical signs of other viral infections such as the deformed wing virus (DWV) or black queen cell virus (BQCV) in apiaries of Buenos Aires province suggest the need of further epidemiological studies in order to determine the prevalence of honeybee viruses in Argentine apiaries and its role in the sudden collapse of colonies.

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