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SHORT COMMUNICATION

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Molecular detection of honeybee viruses in Ecuador

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Abstract

Aim of study: The honeybee, *Apis mellifera,* is one of the most important pollinators in the world. Apicultural activity and pollination services have been affected by the decline in the honeybee population, which may be due to the interaction of multiple risk factors, such as changes in agricultural production, use of pesticides and presence of pathogens. Viruses, in particular, are suspected to be drivers of colony mortality. In this scenario, the aim of this study was to determine the presence of honeybee viruses (IAPV, DWV, SBV, ABPV, BQCV, CBPV) in *A. mellifera* populations using a RT-mPCR assay.

Area of study: Apiaries were situated in Pichincha, Ecuador.

Material and methods: Samples were collected from seventeen apiaries that exhibited mortality but without specific clinical signs. Each sample comprised 15 individuals. After RNA extraction, a multiplex PCR analysis was performed for presence of six viruses (IAPV, DWV, SBV, ABPV, BQCV, CBPV).

Main results: Four of the viruses (ABPV, DWV, BQCV and SBV) were found in co-infections in these colonies, with ABPV and SBV also being found in simple infections.

Research highlights: To our knowledge, this is the first molecular detection of BQCV and SBV in Ecuador. These findings suggest that some of the above viruses could be involved in weakening these colonies.

Additional key words: ABPV, DWV, SBV, BQCV, Multiplex-PCR, *Apis mellifera.*

Abbreviations used: ABPV (Acute Bee Paralysis Virus); BQCV (Black Queen Cell Virus); CBPV (Chronic Bee Paralysis Virus); DWV (Deformed Wing Virus); IAPV (Israeli Acute Paralysis Virus); RT-mPCR (reverse-transcription multiplex polymerase chain reaction); SBV (Sacbrood Virus).

Authors' contributions: Coordinating and designed the experiments: FJR. Performed the experiments: JA, HR and GM. Analyzed the data and wrote the manuscript: MEB. Contributed to the discussion and edited the manuscript: FJR and MLGG. All authors read and approved the final manuscript.

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Introduction

The honeybee, *Apis mellifera,* is of great importance to humanity, mainly because its role as a crop pollinator is vital for the production of food for human consumption. In addition to its pollination service, which improves agricultural production, the honeybee also produces honey, royal jelly, pollen and propolis (Aizen & Harder, 2009). Apicultural activity and pollination services are affected by the decline in honeybee population. In recent decades, colony losses have been reported in Europe, Asia, North America and South America (Todd *et al*., 2007; Ellis *et al*., 2010; Dainat *et al*., 2012; García *et al*., 2019). Colony losses could be related to the interaction of multiple risk factors such as changes in agricultural production, the effects of pesticides, and the effects of several pathogens. Several pathogenic honeybee viruses and honeybee cohabiting pathogenic mites (*Varroa destructor*) have been postulated as the most important factors related to these losses (Potts *et al*., 2010; Meana *et al*., 2017; García *et al*., 2019). To date, 30 viruses have been identified that infect bees worldwide (Remnant *et al*., 2017), most of which are single-stranded positive RNA viruses classified as Picorna-like viruses, belonging to the Dicistroviridae and Iflaviridae families (Remnant *et al*., 2017). Several viruses, including Black Queen Cell Virus (BQCV), Deformed Wing Virus (DWV), Acute Bee Paralysis Virus (ABPV), Chronic Bee Paralysis Virus (CBPV), Kashmire Bee Virus (KBV), Sacbrood Virus (SBV) and Israeli Acute Paralysis Virus (IAPV) have been found infecting honeybees in South America, and in some cases are related to colony mortality (Antúnez *et al*., 2006; Reynaldi *et al*., 2010; Freiberg *et al*., 2012; Rodríguez *et al*., 2012; Sguazza *et al*., 2013; Yañez *et al*., 2014; Riveros *et al*., 2018). Colonies may show clinical signs according to viral infection level and the condition of the bees immune system (Negri *et al*., 2016). While some viruses may not cause specific clinical signs, others can be clearly identified. In adult bees, some of these signs may be trembling, inability to fly, turning black, hair loss, crawling at the entrance of the hive, shivering wings, progressive paralysis and wing deformity (Chen & Siede, 2007; Ribiere *et al.,* 2010). Pupae and larvae can turn black or look like a water-filled sac, and mortality can be observed in queen prepupae (Reynaldi *et al*., 2010; Sguazza *et al*., 2013).

The aim of the present study was to conduct molecular determination of six honeybee viruses in *A. mellifera* populations of Ecuador using a reverse transcription - multiplex polymerase chain reaction (RT-mPCR) assay.

Material and methods

Seventeen colonies were sampled from different apiaries in Pichincha, Ecuador (Fig. 1) in February

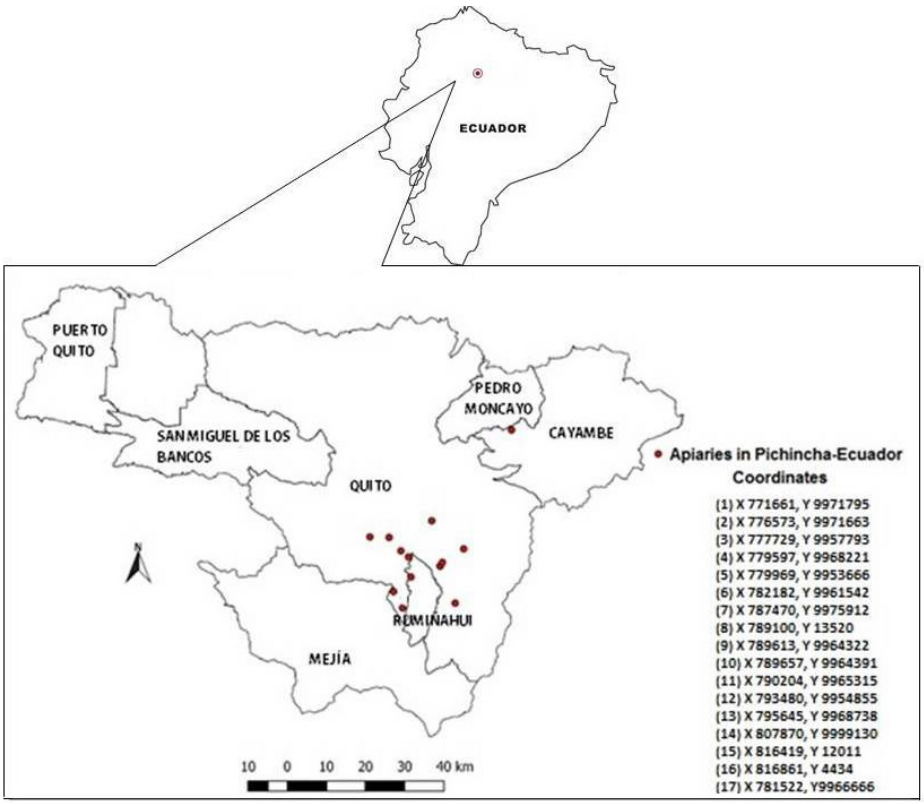

Figure 1. Sample locations, Pichincha, Ecuador.

2017. Each sample comprised 15 individuals and was taken directly from colonies that exhibited mortality without specific clinical signs. The samples were stored at -80°C until they were processed.

Analysis was performed according to Sguazza *et al*. (2013) with modifications. Briefly, each sample was crushed in a mortar with 2 mL of phosphate-buffered saline (PBS). Total RNA was extracted using Trizol® reagent (Invitrogen) and re-suspended in sterile water. Then the RNA yield was calculated in a spectrophotometer based on absorbance at 260 nm and the quality was estimated by using the OD 260/280 absorbance ratio. Synthesis of cDNA was performed using M-MLV (Promega), according to the manufacturer's protocol. The mPCR was performed for the detection of six honeybee viruses (IAPV, DWV, SBV, ABPV, BQCV, CBPV) using specific primers (Sguazza *et al*., 2013; Table 1). The amplification protocol consisted of 95 °C for 5 min (initial denaturation) followed by 35 cycles of 95 °C for 30 sec, 53 °C for 60 sec, 72 ºC for 90 sec, with a final extension of 72 °C for 10 min. The amplification products were analyzed in 2% agarose gel electrophoresis, stained with SYBR Safe ® (Promega). The amplification products were purified using the QIAquick PCR Purification Kit (Qiagen) for sequencing. The obtained amplification products were compared with reference sequences from the GenBank to confirm identity using Basic Local Alignment Search Tool (BLAST) software.

Results and discussion

We detected the presence of four viruses in the studied apiaries: BQCV (536 bp), ABPV (460 bp), SBV (342 bp) and DWV (269 bp). Fifteen out of seventeen samples were positive. We found four co-infections, one of them with three viruses: BQCV–ABPV–DWV (1/17); and the other three with two viruses ABPV–BQCV (1/17); ABPV–SBV (1/17); ABPV–DWV (1/17). Eleven samples were positive for single infections: ten for ABPV and only one for SBV. The viruses IAPV and CBPV were not found in this study (Fig. 2).

Table 1. Sequence of specific primers used in RT-mPCR for the detection of bee viruses (Sguazza *et al*., 2013): ABPV (Acute Bee Paralysis Virus); BQCV (Black Queen Cell Virus); CBPV (Chronic Bee Paralysis Virus); DWV (Deformed Wing Virus); IAPV (Israeli Acute Paralysis Virus); SBV (Sacbrood Virus).

Virus	Primer	Sequence	Product length
IAPV	AIVf IAPVr	GGTGCCCTATTTAGGGTGAGGA GGGAGTATTGCTTTCTTGTTGT	158 bp
DWV	DWVf DWVr	TGGTCAATTACAAGCTACTTGG TAGTTGGACCAGTAGCACTCA	269 bp
SBV	SBVf SBVr	CGTAATTGCGGAGTGGAAAGATT AGATTCCTTCGAGGGTACCTCAT	342 bp
ABPV	AIVf ABPVr	GGTGCCCTATTTAGGGTGAGGA ACTACAGAAGGCAATGTCCAAGA	460 bp
BQCV	BQCVf BQCVr	CTTTATCGAGGAGGAGTTCGAGT GCAATAGATAAAGTGAGCCCTC	536 bp
CBPV	CBPVf CBPVr	AACCTGCCTCAACACAGGCAAC ACATCTCTTCTTCGGTGTCAGC	774 bp

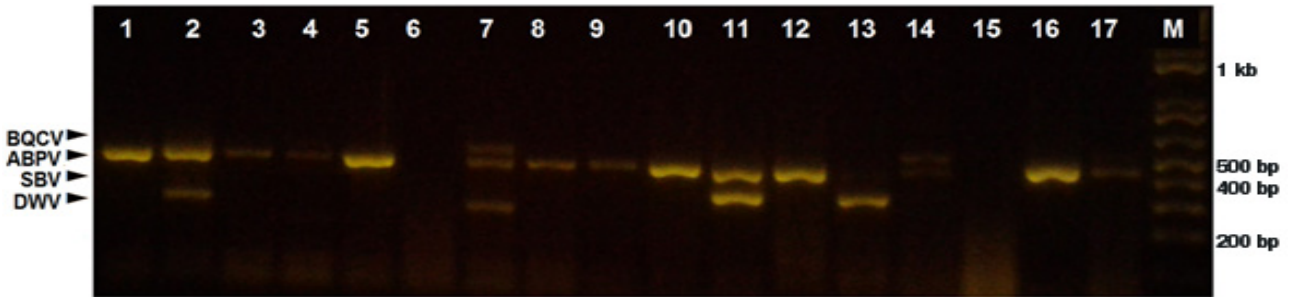

Figure 2. Amplification of bee virus by retro transcription - multiplex polymerase chain reaction (RT-mPCR) from seventeen hives in Pichincha, Ecuador. Wells 1-17: PCR products, M: 1 kb DNA ladder (Promega). The numbers of the wells correspond to the sample locations from Figure 1.

All the sequenced fragments showed 97% homology with ABPV isolate Hungary 1 (Acc. AF486072.2); 98% with BQCV isolate B5-13 polyprotein (Acc. KP223792.1); 96% with DWV isolate R4LY P1R1+ truncated structural polyprotein gene, partial cds (Acc. GU903475.1); and 92% of homology with SBV complete genome (Acc. AF092924.1).

Although our sample size was rather small and ABPV and DWV have been already detected (Avalos *et al*., 2019) in apiaries from Tumbaco (Ecuador) using the mPCR designed by Sguazza *et al.* (2013), this work represents the first molecular detection of BQCV and SBV for Ecuador.

This study found a high virus-prevalence rate, since viruses were found to be positive in 88.2% of the sampled apiaries. Of the seventeen apiaries analyzed, fifteen showed viral presence. Four viruses (ABPV, DWV, BQCV, SBV) were found in co-infections while ABPV and SBV were also found in simple infections. Even though DWV has been reported to be the most prevalent virus worldwide (Natsopoulou *et al*., 2017), in our analysis the most frequent virus was ABPV (14/17). This finding seems to be important, as ABPV is currently associated with the mortality of young and adult bees (Genersch & Aubert, 2010). Worldwide colony losses are associated with several potential factors in interaction, and sometimes viruses play a prevalent role (Stavely *et al.,* 2014; Brutscher *et al*., 2016; Meana *et al*., 2017). In South America, there are records of bee losses that place viruses as one of the candidates of this mortality (Reynaldi *et al*., 2010). Thus, it is conceivable that some of the above honey bee viruses are involved with colony losses in Ecuador.

Our results suggest that viruses may have weakened these colonies. They were probably not the main factor causing deaths in these colonies, but may have been part of a combination of several factors involved in colony losses (Staveley *et al*., 2014). Viruses have the potential to weaken the immune system of bees (Negri *et al*., 2016), making them more susceptible to other risk factors that could lead to colony mortality. Neumann & Carreck (2010) claim that clinical signs and causes of losses may vary among different regions. We therefore believe that the study of viral infections is important and could contribute to the development of strategies to limit colony losses.

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