NOTA CIENTÍFICA

FIRST METAGENOMIC ANALYSIS OF MICROORGANISMS IN HONEY BEES FROM BRAZIL[1]

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ABSTRACT: Alternative approaches including metagenomic and genetic screening can be extremely useful and can accelerate important discoveries related to a current phenomenon wich affect honey bees around the world (characterized by the depopulation or sudden losses of hives). To determine possible causes for the decline in honey bee colonies from southeastern Brazil, and in particular to test the likelihoods of specific predicted causative agent(s) for this condition, a diverse set of analyses were proposed. These analyses form part of a more extensive Epidemiological Evaluation Program for bees in southeastern Brazil. Here we present an overview about part of this Program. Three viruses were identified (Acute Bee Paralysis Virus - ABPV, Black Queen Cell Virus - BQCV, and Deformed Wing Virus - DWV) in 1,920 analyzed bees, and a multiplex RT-PCR assay was developed and validated. Although strategy adopted to analyze data in details is still being evaluated and prepared by the authors, the first results indicated that ca. 26 % of the samples carried Israeli Acute Paralysis Virus (IAPV), and some isolates for this virus were found. *Nosema ceranae*, *Melissococus pluton*, *Leptomonas apis*, and *Spiroplasma apis* were also detected. Pathological, epidemiological, and widespread studies remain to be conducted once no single pathogen seems overly predominant in declining bee colonies.

Key words: Africanized honey bees, colony collapse disorder, pathogens, RT-PCR, real-time RT-PCR.

PRIMEIRA ANÁLISE METAGENÔMICA DE MICROORGANISMOS EM ABELHAS MELÍFERAS ORIUNDAS DO BRASIL

RESUMO: Abordagens alternativas, incluindo análises genéticas e metagenômicas, podem ser extremamente úteis para descobertas relacionadas ao atual fenômeno que vem afetando as abelhas melíferas no mundo (caracterizado pelo declínio populacional ou perda súbita de colméias). Para identificar as possíveis causas envolvidas com o declínio populacional em colméias da região sudeste do Brasil e, em particular, para testar as semelhanças com possíveis agentes causadores envolvidos com tal fenômeno, diversas análises foram propostas. Tais análises fazem parte de um extenso Programa de Avaliação Epidemiológica para abelhas oriundas da região sudeste do país e parte desse Programa e de seus resultados encontram-se aqui apresentados. Três vírus foram identificados (Acute Bee Paralysis Virus - ABPV, Black Queen Cell Virus - BQCV, and Deformed Wing Virus - DWV) em 1920 abelhas analisadas e um ensaio multiplex RT-PCR foi

desenvolvido e validado. Embora certas estratégias adotadas para análise dos dados com maiores detalhes encontrem-se ainda em preparação, os primeiros resultados indicaram que aproximadamente 26% das amostras analisadas encontravam-se infectadas pelo vírus Israeli Acute Paralysis Virus (IAPV) e diferentes isolados de tal vírus foram identificados. *Nosema ceranae*, *Melissococus pluton*, *Leptomonas apis*, and *Spiroplasma apis* foram também detectados. Estudos patológicos, epidemiológicos e de dispersão necessitam ser conduzidos, uma vez que nenhum patógeno, em particular, parece ser predominante nas colônias afetadas pelo mencionado declínio.

Palavras-chave: abelhas africanizadas, patógenos, RT-PCR, RT-PCR em tempo real, colony collapse disorder.

INTRODUCTION

In a strict sense, metagenomic surveys provide data to understanding the genetic components of entire ecological communities. Recently, metagenomic efforts have focused on describing the microbial components of ecosystems ranging from the open ocean to the human digestive tract. In the last few years this cultivation-independent approach has produced many exciting discoveries, and especially the identification of many organisms that are either new to science or not previously associated with a given ecosystem or biological sample. The feasibility and applicability of modern molecular techniques in identifying organisms is evident and such techniques are being used widely. A current phenomenon that is affecting honey bees around the world, characterized by the depopulation or sudden losses of hives, remains without a recognizable underlying cause, despite much effort using many analytic strategies. As such, alternative approaches including metagenomic and genetic screening, can be extremely useful and can accelerate important discoveries (Rondon *et al*., 2000; Gillespie *et al*., 2002; Schloss and Jo Handelsman, 2003; Handelsman, 2004; Edwards and Rohwer, 2005; Xu, 2006; Eisen, 2007).

Honey bees, like other organisms, face disease agents including viruses, bacteria, fungi, protozoa, parasitic mites and nematodes (Bailey and Ball, 1991; Ellis and Munn, 2005). One factor in the success of Africanized honey bees (AHBs, hybrid resulted from crosses between European and African honey bee subspecies) across the Americas seems to be higher levels of resistance toward mites and other pathogens (Aumeier, 2001; Guzmán-Novoa *et al*., 1999; Mondragón *et al*., 2005; Moretto and Mello Jr., 1999; Moretto and Mello Jr., 2001; Rosenkranz, 1999; Vandame *et al*., 2002). Nevertheless, in the last five years, decreased worker bee populations can be observed in some areas of southeastern Brazil. These losses tend to occur between March and July, and have in some cases been correlated with specific brood symptoms and, sometimes, few dead adult bees in front of the colony. Nevertheless, the defining trait observed by beekeepers is simply the occurrence of significant and unexpected production losses. Consequently, in Brazil terms like "depopulation" or "decline" best describe the observed problem. This contrasts somewhat with Colony Collapse Disorder (CCD), a recent phenomenon observed in the USA, and defined by: "sudden loss of the colony's adult population with very few bees found near the dead colonies; several frames with capped brood indicating that colonies were relatively strong shortly before the loss of adult bees; food reserves that have not been robbed, despite living colonies in the area, suggesting avoidance of the dead colony by other bees; minimal evidence of wax moth or small hive beetle damage; a laying queen often present with a small, 100-bees, cluster of young attendants" (Pettis, 2007).

To determine possible causes for the decline in honey bee colonies from Brazil, and in particular to test the likelihoods of specific predicted causative agent(s) for this condition, a diverse set of analyses were proposed. Bees were sampled in a specific area in the Southeast, where the problem has been observed more evident in the last years, in the municipality of Altinópolis, São Paulo State. These analyses form part of a more extensive Epidemiological Evaluation Program for bees in southeastern Brazil. All analyses converged to the same purpose: to evaluate the prevalence of various parasites and pathogens of brood and adults bees, or other causes (like pesticides and toxic nectar and pollen), which could be involved directly or indirectly in such losses. Based on the obtained results, the propose is to develop control methods without the use of chemotherapy, to make sure the apicultural products in Brazil will remain viable productive without drugs.

Here we present an overview about part of this Program, which is being addressed in collaboration among Brazilian and American scientists from USDA, Beltsville, USA. Initially, and considering the fact that viruses have emerged as one of several candidates for these losses, we developed a study termed "Virus infections in Brazilian honey bees". In this study different viruses were detected and a multiplex RT-PCR assay was validated (TEIXEIRA *et al*., 2008). After that, searching for similarities between the problem that is happening in the USA (CCD) and the problem that is severely impacting beekeepers in southeastern Brazil we evaluated the presence of Israeli Acute Paralysis Virus (IAPV) in those samples, since IAPV was detected in 83% CCD-affected honey bee colonies in the USA, and could be involved in such phenomenon maybe like a good marker for that (COX-FOSTER *et al*., 2007). Additionally, the presence of others microorganisms was evaluated (*Nosema apis*, *Nosema ceranae*, *Paenibacillus larvae*, *Melissococus pluton*, *Spiroplasma apis* and *Leptomonas apis)*, and lastly, applying knowledge about honey bee immune gene expression, we evaluated genes related to antimicrobial, melanization and recognition processes, as well.

MATERIAL AND METHODS

Africanized adult honey bees were sampled from 200 colonies from 10 apiaries (20 colonies/apiary and 20 bees/colony) located in a representative area where the problem has been observed more evident in the last five years (Altinópolis, São Paulo, Brazil) where the abnormality has been observed in the last five years, during the autumn time in 2007. Prior to shipment to the United States, to avoid RNA degradation all samples were grounded in RNAlater® Tissue Collection (RNA stabilization solution, Ambion). After shipment time (~3 days) at room temperature all samples were stored at -80ºC. RNA isolation, cDNA synthesis, primers designed, amplicon sizes, amplification conditions, sequences, and other details related with virus identification and multiplex RT-PCR assay were described in TEIXEIRA *et al.* (2008). Nucleotide sequences of the amplified products were determined and confirmed to be the expected species using BLAST at the US-NIH National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). In order to address if IAPV nucleic acid was present, PCR primers were the same used in CHEN and EVANS (2007; IAPV-F1a: GCGGAGAATATAAGGCTCAG, IAPV-R1: CTTGCAAGATAAGAAAGGGGG). Strategy adopted to analyze data in details is still being evaluated and prepared by the cooperative group (TEIXEIRA *et al*., unpublished data). DNA was extracted from 192 compound samples by placing 5 µL of the RNA-Later suspensisons above into 100 µL of 5% Chelex-100 (Bio-Rad). The solution was incubated at 95ºC for five minutes and the concentration and purity of the resulting DNA was evaluated using a NanoDrop machine (Thermo Scientific). Quantitative PCR assays were conducted for six additional pathogens (the bacteria *Melissococcus pluton* and *Paenibacillus larvae*, and *Spiroplasma apis* the microsporidia *Nosema apis* and *Nosema ceranae*, and the trypanosome *Leptomonas apis*. Oligonucleotide primer pairs from the two primer species were as published in CHEN *et al.* (2008). Bacterial primers used for *Paenibacillus larvae* (PLS18, EVANS, 2006; F: 5'-TTCACGGCTAACAAAATTAAACA-3', R: 5'-TTCGCAGAAGTTCCGGTTAC-3'), *Melissococcus pluton* (F 5'-ACGCCTTAGAGATAAGGTTTC-3' R 5'-GCTTAGCCTCGCGGTCTTGCGTC-3'), and *Spiroplasma apis* (F: 5'-CTTTTAGTTTGACGGTACCTTACCAG-3', R: 5'-AACCGCCTACGCACCCTAT-3'). For *Leptomonas apis* was designed a primer pair (TrypITS1 F: 5'-GTCGTTGTTTCCGATGATGG-3', TrypITS1 R: 5'-CCGTTTGCGTTCAAAGATTC-3') to generate a 120 bp product, based on a partial rRNA sequence generated in our initial screening of Brazilian bees (Genbank accession pending). PCR reaction mixtures (25µL) consisted of a primer concentration of 0.2µM each, 1 U Taq with proscribed 1× buffer (Roche Applied Sciences), a final concentration of 1 mM dNTP mix and 2µL of DNA template (~ 90 ng). Reactions were carried out with a thermal protocol of 96ºC for 2 min., then 3 cycles of 96ºC for 30 sec., 60ºC for 30 sec. (-1ºC/Cycle), 65ºC for 1 min., followed by 35 cycles of 96ºC for 30 sec., 56ºC for 30 sec., 65ºC for 1 min, and a final extension at 65ºC for 2 min. Positive and negative controls were included. PCR products were visualized on a 1.7% (w/v) agarose gel containing ethidium bromide. Nucleotide sequences of the amplified products were determined by Big-Dye sequencing (Applied Biosystems) and confirmed using BLAST at the National Center for Biotechnology Information (NCBI). Conditions described by EVANS (2006) were adopted as to develop real-time RT-PCR like an initial diagnosis for viruses (except for IAPV), as to develop a quantitative-PCR array to measure

transcript levels for 192 compound samples of 10 bees each and a set of four honey bee immune-genes selected validated previously (EVANS, 2006). The four-set genes' included in this study comprises different categories of gene function in bees: immune end product - abaecin F (5'- CAGCATTCGCATACGTACCA - 3') R (5'- GACCAGGAAACGTTGGAAAC - 3'), and hymenopt F (5' - CTCTTCTGTGCCGTTGCATA - 3') R (5'- GCGTCTCCTGTAATTCCATT - 3'); pathogen recognition - Bgluc2 F (5'- TTCTTTTCATGACGAATTGTTTT- 3') R (5'- CCGTTCGAATGTGGTGAAC - 3'; CyP4G11 F (5'- CAAAATGGTGTTCTCCTTACCG- 3') R (5'- ATGGCAACCCATCACTGC- 3').

As in EVANS and PETTIS (2005), threshold cycle (Ct) numbers for the immune-peptide gene abaecin were subtracted from the RPS5 threshold used as honey bee control (housekeeping gene) to normalize data for each sample prior to statistical analyses (RPS5 F: 5'- AATTATTTGGTCGCTGGAATTG-3', R: 5'- TAACGTCCAGCAGAATGTGGTA-3'). Bee samples, RNA extraction and cDNA synthesis were the same as used in TEIXEIRA *et al*. (2008). Transcript levels were quantified for Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Deformed Wing Virus (DWV), *N. apis*, and *N. ceranae*. To confirm the apparently low levels or absence of *N. apis*, we also screened for *N. apis* using PCR and a gel-based assay as described in CHEN *et al*., 2008. Statistical analysis were performed using the SAS JMP software package. Analyses of variance using the CT values as dependent variables were carried out since these showed a more normalized distribution that did the transformed (biological) variables.

RESULTS

While screening for the six more common viruses in adult honey bees from Brazil (Acute Bee Paralysis Virus - ABPV, Black Queen Cell Virus - BQCV, Deformed Wing Virus - DWV, Chronic Bee Paralysis Virus - CBPV, Kashmir Bee Virus - KBV, and Sacbrood Virus - SBV), nucleic acid for the three first viruses were identified (ABPV, BQCV, and DWV) in 1,920 analyzed bees (TEIXEIRA *et al*., 2008). In order to detect simultaneously these three target viruses (ABPV, BQCV, and DWV) authors developed and validated a multiplex RT-PCR assay, which generated unique amplicon sizes of 700 bp, 500 bp and 129 bp., respectively. Products of the expected size and sequence were obtained (Genbank accession numbers EU292210, EU292211 and EU292212 for ABPV, BQCV, and DWV, respectively). In relation to the prevalence of the virus, 52, 71 and 39 samples were positive for ABPV, BQCV, and DWV, respectively. Forty three samples were co-infected colonies 15 with ABPV and DWV, 19 with BQCV and DWV, and 21 with ABPV and BQCV. Among them only 6 with all of three viruses. Among adult bee samples analyzed from brood area, our first results indicated that ca. 26% carried IAPV.

Transcript abundances of immune-response genes in honey bees relation to *Nosema ceranae*, *N. apis*, ABPV, BQCV, and DWV are shown in the Figure 1. The colors, ranging from green to red, corresponding to relatively low levels to high levels of expression, while dark ray cells represent moderate expression levels. *N. apis* was essentially absent from our analyses (green cells). There is not a strong trend in terms of immune-gene expression in samples collected from declining versus healthy colonies. Correlations between the Ct values of pathogens were low (from -0.005 to 0.19), although significant ($P< 0,05$) in some cases (ABPVxBQCV, ABPVxDWV, *Leptomonas apis*xABPV, and *Nosema ceranae*xDWV). These few cases may indicate synergistic impacts of pathogen species.

In RT-PCR gel assays, no samples were positive for either *N. apis* or *P. larvae*. Only two samples were infected with *M. pluton*. *Leptomonas apis* nucleic acid was present in 36/94 samples that were screened and sequenced. *Spiroplasma apis* was detected in 68 samples, but these primers co-amplified other 18S sequences, and so need further analysis. Correlations between the Ct values of pathogens are shown in Table 1.

DISCUSSION

Three different bee viruses, ABPV, BQCV and DWV, were found during a screening of RNA's from 1,920 individual adult bees from the municipality of Altinopolis, São Paulo State, Brazil, and a multiplex RT-PCR assay was developed and standardized for these target viruses, validated to be precise and sensitive (TEIXEIRA *et al*., 2008). The authors demonstrated in this first published assay using molecular techniques in Brazilian bees that viruses are considerably less prevalent compared to elsewhere, including when compared with a previous South American evaluation from Uruguay

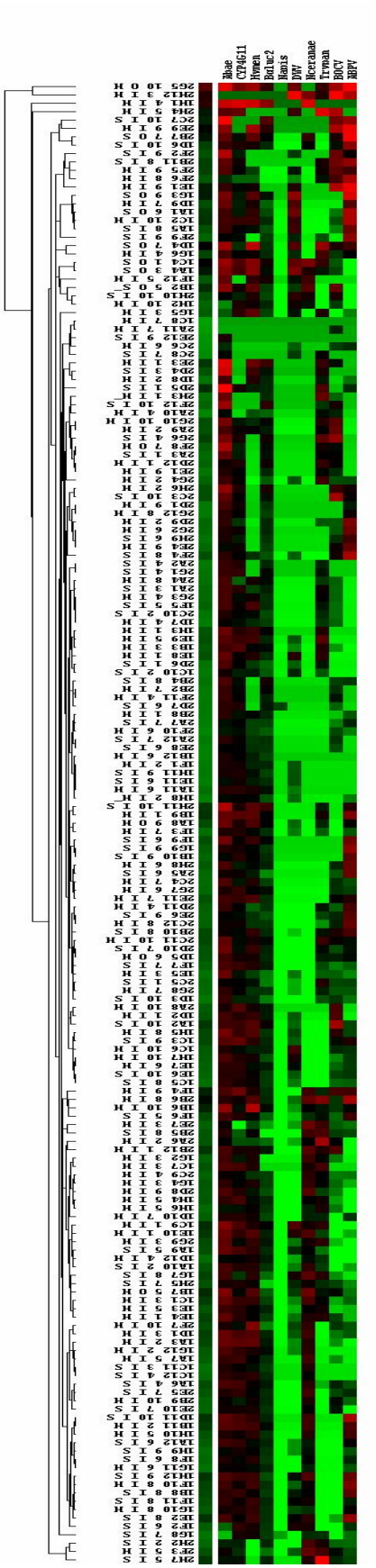

Figure 1. Array diagram showing expression patterns of honey bee immune genes and pathogens in relation to sample disease status (S = "sick", H = "healthy"), and collection site (I = inside bees, O = outside bees) for colonies collected in 10 apiaries (as in Teixeira *et al.*, unpublished data). Genes surveyed (vertical names) are the honey bee genes Abaecin, cytochrome P450, hymenoptaecin, and B-glucan recognition protein and bee pathogens *Nosema apis*, Deformed wing virus, *N. ceranae*, trypanosome, Black queen cell virus and Acute bee paralysis virus.

Table 1. Correlation matrix of the Ct values of the pathogens

	BQCV	DWV	*N. ceranae*	*Leptomonas apis*
ABPV	0.146*	0.189*	0.035	-0.160*
BQCV	--	0.072	-0.041	0.097
DWV	--	--	0.002	-0.005
N. ceranae	--	--	--	0.118

* P< 0.05

(ANTÚNEZ *et al*., 2006). Previously, MESSAGE *et al*. (1996), using double-immune diffusion, detected the presence of APV (=ABPV), BQCV, FV (Filamentous Virus), and CWV (Cloud Wing Virus) in Brazil, but not DWV, CBPV, KBV, and SBV.

Although our IAPV data set is still under evaluation, we found at least several isolates for this virus that are distinct from other discistroviruses. We could observe that IAPV from Brazil seems to be different in average 2.18 % (by 1.54 % - 3.11 %) from isolates from Israel (MAORI *et al*., 2007) and differs on average 2.44 % (by 0 % - 4.94 %) from isolates in the U.S. (CHEN and EVANS, 2007). Additionally we found an interesting diversity among Brazilian samples (by 0 % to 4.23 %, and 1.8 % in average), considering the total alignment length of 462 base pairs. *Leptomonas apis*, a trypanosome parasite of bees, was the microorganism most frequently found in all analyzed adult bee samples. This species appears not to be strongly tied to disease, either by presence/absence tests or quantitative PCR (Figure 1). It is important consider that all insect species are known to harbor a rich and complex community of microorganisms in their guts and other body regions. This microbiota participates in many types of interactions ranging from pathogenesis to obligate mutualism (DILLON and DILLON, 2004; DHARNE *et al*., 2006).

It is interesting to mention that part of the symptoms described by beekeepers and scientists for CCD are observed in Brazil when the honey bees collect pollen from *Stryphnodendron* spp. In this case strong colonies abandon their hives in a few days leaving strong amounts of brood with Brazilian Sac Brood-like disease and food reserves. Normally the strongest colonies are more affected because necessity of pollen is higher. On the other hand some of these symptoms are quite different of CCD because strong brood mortality is observed. The larvae fail to pupate and remain stretched on their backs, with their heads towards; fluid accumulates between the body of the diseased larvae and its unshed skin (CARVALHO and MESSAGE, 2004). Such observation permit us wonder about possible involvement of toxic nectar or pollen in the actual abnormality. Possible exposure to other toxic compounds (like some pesticides that have been extensively adopted recently) or a combination of different causes should be considered as well.

While the results presented here confirm the presence of diverse microorganisms carried by AHB samples from Brazil, pathological, epidemiological, and widespread studies remain to be conducted. In addition, no single pathogen seems overly predominant in declining bee colonies.

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