

Simultaneous detection of *Nosema* spp., *Ascospaera apis* and *Paenibacillus larvae* in honey bee products

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Abstract

Honey bees are responsible for pollinating many native and cultivated plant species. These insects can be affected by many pathogens, including fungi and bacteria, both of which can form spores that are easily dispersed within the colony by means of the stored products, among other routes. The objective of this study was to develop a method to detect spores of the honey bee pathogens *Nosema apis*, *Nosema ceranae*, *Ascospaera apis* and *Paenibacillus larvae* in samples of honey, bee pollen and royal jelly. The method was standardized for each product individually, and then analyzed by monoplex and multiplex PCR, which showed the same detection thresholds: 1.25 spores/mL of honey for *N. ceranae*; 7.5 spores/mL of honey for *A. apis*; and 0.4 spore/mL of honey for *P. larvae*, respectively. The standardized technique was effective and rapid for the detection of these pathogens in bee products and can be used for the establishment of official methods of sanitary control of bee products, considering the growing national and international trade of these products and the movement or migration of colonies between regions.

Keywords

Apis mellifera, Bee pathogens, Honey, Pollen, Royal Jelly, Detection of spores

Introduction

The honey bee, *Apis mellifera*, is essential to global agriculture by pollinating a wide range of food crops, besides supplying economically important products (Evans and Schwarz 2011). These insects can be affected by various pathogens (Bailey and Ball 1991), including the bacterium *Paenibacillus larvae* and the fungi *Ascosphaera apis*, *Nosema apis* and *Nosema ceranae*. These fungi stand out for their ease of transmission in the form of spores, which can remain viable in the environment for many years, transmitting diseases between colonies and among their individuals (Shimanuki and Knox 1994).

Within the colony, products like honey, pollen and royal jelly are susceptible to contamination by these pathogens, mainly through storage in honeycomb and trophallaxis (OIE 2014). In addition, some negligent hygienic practices by beekeepers, such as reuse of apiculture materials (Van der Zee 2010), exchange of combs containing the remains of diseased broods (OIE 2014), and reuse of contaminated wax (Malone and Gatehouse 1998; OIE 2014), among other practices (Higes et al. 2008; Aronstein and Murray 2010), are indicated as important routes for dissemination of these pathological agents (Hale and Menapace 1980; Higes et al. 2008; Giersch et al. 2009). For this reason, research has been carried out to develop methods to detect pathogens in bee products, mainly involving the use of polymerase chain reaction (PCR) (de Graaf et al. 2013).

Multiplex PCR is used if more than one pathogen is amplified simultaneously in a single reaction, resulting in considerable savings of both time and effort (Sigh et al. 2000), besides reducing costs of reagents and allowing fast analysis of a large number of samples. Thus, molecular biology techniques such as multiplex PCR are important tools for the detection of pathogens.

The aim of this study was to develop a method to detect spores of *N. apis*, *N. ceranae*, *A. apis* and *P. larvae* in honey, bee pollen and royal jelly samples.

Materials and methods

Solutions containing spores of *Nosema ceranae* and *Ascosphaera apis* were prepared from naturally contaminated bees according to the protocols described by Teixeira and Message (2010) and Cantwell (1970). For the *Paenibacillus larvae* spore suspension, we were used a solution of ATCC 9545, supplied by the National Agriculture Laboratory - Ministry of Agriculture. The solutions were inoculated in different quantities of sterile bee products, previously submitted to cobalt irradiation, receiving an ion beam dose of approximately 960 Gy/10 cm: honey (5 mL, 10 mL and 20 mL), pollen (0.5 g, 1 g, 5 g and 10 g) and royal jelly (0.5 g, 1 g, 5 g and 10 g), which were submitted to different analytic processes (homogenization, filtration and centrifugation) to test the spore concentrations. The presence of pathogens was confirmed by PCR and sequencing before the tests.

For detection of the pathogens, the samples were weighed (10 g for pollen, 5 g for royal jelly and 20 mL for honey) in sterile tubes and diluted in 40 mL of sterile distilled water. The samples of pollen were diluted in 45 mL of sterile distilled water. The samples were vigorously homogenized and centrifuged at $12,500 \times g$ for 40 min. The supernatant was discarded and the pellet was resuspended in 1 mL of sterile distilled water, with subsequent homogenization and centrifugation at $10,000 \times g$ for 20 min. The supernatant was again discarded and the pellet was submitted to DNA extraction using the Qiagen DNeasy® Plant Mini Kit, following the manufacturer's instructions. In the case of bee pollen, after homogenization the samples were filtered with Whatman 1 filter paper using a vacuum pump and then the same procedure was followed as for the samples of honey and royal jelly.

Monoplex and multiplex PCR were carried out following Puker (2011) to confirm the detection of each pathogen in the products analyzed. These reactions were performed with a volume of 20 μL , composed of 10 μL of GoTaq® Green (Promega), 8 μL of DNase free water, 1 μL of sample DNA and 1 μL of each primer (10 μM) (Piccini et al. (2002), Murray et al. (2005) and Martin-Hernandez et al. (2007) for *P. larvae*, *A. apis* and *N. apis/N. ceranae*, respectively). All analyses were carried out with positive and negative controls. Spores of *N. ceranae* removed from forage bees naturally infected with the microsporidian were used as positive control. The positive controls of *N. apis* was sent by the Bee Research Laboratory – USDA, considering the low prevalence of the pathogen in Brazil (0.39%) (Teixeira et al. 2013), causing it often not to be detected (Santos et al. 2014). As positive control of *P. larvae*, spores of ATCC 9545 supplied by Lanagro were used.

Decreasing spore concentrations were tested (2500, 250, 100, 50, 25 spores of *Nosema ceranae*; 3000, 300, 150, 75 spores of *Ascospaera apis*; 1000, 100, 10, 8, 4, 1 spores of *Paenibacillus larvae*), until reaching the minimum detectable number, in order to identify the technique's sensitivity. All the analyses were performed at the Honey bee Health Laboratory of São Paulo State Agribusiness Technology Agency (LASA/APTA), located in Pindamonhangaba, São Paulo.

Results

Multiplex PCR allowed the simultaneous detection of *N. ceranae*, *A. apis* and *P. larvae* in different bee products, and the amplified product for each pathogen presented the expected fragment lengths (218 bp, 485 bp and 700 bp, respectively). Threshold values were the same for the monoplex and multiplex reactions (Figure 1). In samples of honey, we found 1.25 spores/mL of honey for *Nosema ceranae* (218 bp), 7.5 spores/mL for *Ascospaera apis* (485 bp) and 0.4 spore/mL for *Paenibacillus larvae* (700 bp). For bee pollen we found 2.5 spores/g of pollen for *N. ceranae* (218 bp), 15 spores/g for *A. apis* (485 bp) and 0.8 spore/g for *P. larvae* (700 bp). For samples of royal jelly we found 5 spores/g of royal jelly for *N. ceranae* (218 bp), 30 spores/g for *A. apis* (485 bp) and 1.6 spores/g for *P. larvae* (700 bp).

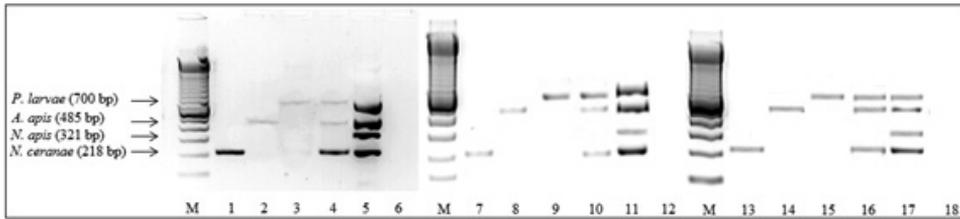


Figure 1. Agarose gel (2%) stained with SYBR® Safe of the monoplex and multiplex PCR products referring to the detection thresholds of the pathogens in honey bee products. M: 100 bp marker. **1** 2.5 spores/g of pollen for *Nosema ceranae* **2** 15 spores/g of pollen for *Ascosphaera apis* **3** 0.8 spore/g of pollen for *Paenibacillus larvae* **4** multiplex PCR for *N. ceranae* (2.5 spores/g of pollen), *A. apis* (15 spores/g of pollen) and *P. larvae* (0.8 spore/g of pollen) **5** multiplex positive controls for *N. ceranae* (218 bp), *N. apis* (321 bp), *A. apis* (485 bp) and *P. larvae* (700 bp) **6** negative control. M: 100 bp marker **7** 1.25 spores/mL of honey for *N. ceranae* **8** 7.5 spores/mL of honey for *A. apis* **9** 0.4 spore/mL of honey for *P. larvae* **10** multiplex PCR for *N. ceranae* (1.25 spores/mL of honey), *A. apis* (7.5 spores/mL of honey) and *P. larvae* (0.4 spore/mL of honey) **11** multiplex positive controls for *N. ceranae* (218 bp), *N. apis* (321 bp), *A. apis* (485 bp) and *P. larvae* (700 bp) **12** negative control. M: 100 bp marker **13** 5 spores/g of royal jelly for *N. ceranae* **14** 30 spores/g of royal jelly for *A. apis* **15** 1.6 spores/g of royal jelly for *P. larvae* **16** multiplex PCR for *N. ceranae* (5 spores/g of royal jelly), *A. apis* (30 spores/g of royal jelly) and *P. larvae* (1.6 spores/g of royal jelly) **17** multiplex positive controls for *N. ceranae* (218 bp), *N. apis* (321 bp), *A. apis* (485 bp) and *P. larvae* (700 bp) **18** negative control.

Because *N. apis* occurs in very low prevalence (Teixeira et al., 2013), and is difficult to detect in naturally infected samples, spores of this species were not used in the standardization. We believe the detection threshold would be the same as that obtained for *N. ceranae* (Martín-Hernandez et al. 2007), where the amplification when compared to the positive control worked perfectly.

Discussion

Detection of *Nosema apis*, *N. ceranae*, *Ascosphaera apis* and *Paenibacillus larvae* in samples of bee products has been reported (Cox-Foster et al. 2007, Higes et al. 2008, Giersch et al. 2009), but few studies have reported the detection limits.

Puker (2011) centrifuged samples of honey at 10,000 × g for 40 min and reported detection limits of 10 and 100 spores/mL of honey for *Ascosphaera apis* in monoplex and multiplex reactions, respectively, 100 spores/mL of honey for *Nosema ceranae*, and 10 spores/mL of honey in monoplex and multiplex reactions for *P. larvae*. Our results showed lower limits of detection (1.25 spores / mL honey for *N. ceranae*, 7.5 spores / mL for *A. apis* mel and 0.4 spores / mL honey for *Paenibacillus larvae*, which can be attributed to the increase in the centrifugation speed and time.

Some studies have presented detection limits that corroborate this idea. Piccini et al. (2002) obtained a detection limit of 32 spores/mL of honey, analyzing 10 mL of honey and centrifuging the samples at 6,000 × g for 20 min. D'Alessandro et al. (2006)

also analyzed honey and found a detection limits of 20 spores/reaction, using 20 mL of honey and centrifuging the samples at $6,000 \times g$ for 45 min. In these studies, the limits of detection decreased when the spin time was increased.

Although the technique presented here is similar to those employed by other authors, our tests showed that the samples centrifuged below $12,000 \times g$ did not undergo sedimentation, and consequently a considerable number of spores were still present in the supernatant.

To assure that no spores remained in the supernatant, besides performing a centrifugation at $12,000 \times g$ for 40 min, we also submitted the resuspended pellet obtained to new centrifuging at $10,000 \times g$ for 20 min, for subsequent extraction of DNA. This factor is likely responsible for the technique's sensitivity.

For detection of the bacterium *Paenibacillus larvae*, various methods have been described, mainly using growth in culture medium and PCR (de Graaf et al. 2013; OIE 2014). The Manual of OIE (2014) describes detection of spores in suspension in samples of pollen and royal jelly, employing centrifuging at $6,000 \times g$ during 30 min. This centrifuge force can be considered low compared to that used here, which may have left spores in the supernatant, thus hindering efficient detection of the pathogens contained in the samples.

According to the OIE (2014), for the analysis of *Paenibacillus larvae* in pollen, 1 g of the product in 10 mL of sterile distilled water or PBS should be filtered through Whatman 1 filter paper for subsequent microbiological analysis. We also suggest this filtration of pollen samples, because without this step the samples still contained a large quantity of material after centrifuging, which can have a negative effect on extraction of the genetic material and also on the PCR reactions.

Microbiological techniques have also been used to detect *Paenibacillus larvae*, but it is important to consider the time for analysis and efficacy in detecting this bacterium. According to the official technique specified in Brazil (Brasil 2003), it is necessary to wait five days for colony growth in PLA culture medium, plus over three days for confirmatory tests. In other suggested microbiological techniques, it is necessary to incubate the bacteria in culture medium for 6, 7 and 8 days (de Graaf et al. 2013; OIE 2014). The time frame thus varies from 5 to 11 days, placing a significant burden on beekeepers.

The protocol developed showed important results when used to analyze samples of bee products marketed in São Paulo state (in preparation), supporting the idea that PCR is a fast and reliable technique to diagnose pathogen infections (Martin-Hernandez et al. 2007; de Graaf et al. 2013).

The multiplex PCR method offers a significant cost-saving advantage, especially when large numbers of samples are analyzed (Sguazza et al. 2013). Furthermore, multiplex PCR is able to detect multiple target DNA sequences in a single reaction, with the simultaneous identification of two or more kinds of pathogens, simplifying the workflow and processing time, which are significantly reduced (Bilgic et al. 2013), among other advantages (Edwards and Gibbs 1994).

The protocol presented here is useful to detect simultaneously *Nosema apis*, *Nosema ceranae*, *Ascospaera apis* and *Paenibacillus larvae* in samples of honey, pollen and royal jelly and can support definition of official methods for surveillance actions.

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