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Spores of *Paenibacillus larvae*, *Ascosphaera apis*, *Nosema ceranae* and *Nosema apis* in bee products supervised by the Brazilian Federal Inspection Service

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ABSTRACT

Due to their ecological and economic importance, honey bees have attracted much scientific attention, which has intensified due to the recent population decline of these insects in the several parts of the world. Among the factors related to these patterns, infection by pathogens are the most relevant, mainly because of the easy dissemination of these microorganisms. Although no zoonotic diseases are associated with these insects, the presence of infectious agents in bee products should still be considered because they play a role as disease dispersers, increasing the risk to animal health. Because of the possibility of dispersion of pathogens via bee products, this work aimed to identify the presence of spores of the pathogens *Paenibacillus larvae*, *Ascosphaera apis* and *Nosema* spp. in samples of honey, pollen and royal jelly that are registered with Brazil's Federal Inspection Service (S.I.F.) and commercially available in the state of São Paulo. Of the 41 samples of bee products analyzed, only one showed no contamination by any of these pathogens. *N. ceranae* and *P. larvae* had the highest prevalence considering all the samples analyzed (present in 87.80% and 85.37% of the total, respectively), with *N. apis* present in 26.83% and *A. apis* present in 73.17% of the samples. These results provide support for the formulation of government regulations for sanitary control of exotic diseases by preventing dispersion of pathogens, including through illegal importation, since local and international trade and the transfer of colonies between regions play important roles in the dispersion of these microorganisms.

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Introduction

Honey bees are important to the sustainability of many natural and agricultural systems due to their role in pollination (FAO, 2004; Kremen, 2004; Yue et al., 2008; Garcia-Gonzalez and Genersch, 2013), contributing to >10% of world food production (FERA, 2013).

Many microorganisms are associated with honey bees and their products. The main sources of contamination are the environment (e.g. pollen, nectar and dust), followed by their digestive tracts (Snowdon and Oliver, 1996). Some infectious agents can lead to the development of diseases, including viruses, bacteria, fungi, protozoa, nematodes and parasitic mites (Bailey and Ball, 1991; Ellis and Munn, 2005). Pathogens can also be present in the bee products such as honey, pollen and royal jelly from infected colonies. The presence of infectious agents in these products should be considered because products act at disease dispersers, increasing the risk to animal health.

Honey, pollen and royal jelly usually do not undergo transformations before sale. Thus, factors inherent in their compositions, whether intrinsic (e.g., water, pH, presence of inhibitory compounds) or extrinsic (environmental), will influence the survival of microorganisms in these bee products (Franco, 1996). Spores of pathogens are resistant to adverse conditions (Dobbelaere et al., 2001; OIE, 2016), and thus pose a potential risk to bee health.

In Brazil, the importation of bees and their products is permitted as long as, specific health standards are met (Brasil, 2013), in

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order to avoid introducing diseases or genetic variations of existing pathogens (different haplotypes or strains).

The main bottleneck related to beekeeping in Brazil is the absence of official laboratories and techniques that attest to the absence of pathogens in bee products, except microbiological analysis to identify *Paenibacillus larvae* spores in honey, using traditional microbiological procedures, which can also be used to analyze other bee products (Brasil, 2003). However, this technique is time-consuming taking almost eight days to obtain the final result. Molecular approaches have been reported as powerful tools for identification of microorganisms associated with bees, with reduction of time and optimization of resources (Teixeira et al., 2008).

Among the diseases that affect bees, American foulbrood (AFB), chalkbrood and nosemosis are caused by infectious agents that spore form is part of their life cycle. AFB, caused by the gram-positive bacterium *P. larvae*, is the most severe disease in terms of losses caused to beekeepers in several countries (Johnson, 2007). This disease is highly contagious and lethal (Garcia-Gonzalez and Genersch, 2013), with bacterial spores causing infection of bee larvae (Yue et al., 2008).

In southern Brazil Schuch et al. (2003) isolated for the first time bacterial spores in honey from hive combs without clinical signs of the disease. In 2006, the first outbreak of AFB occurred in this region, which was notified to the World Organization for Animal Health (OIE) and controlled by the Brazil's Official Veterinary Service, with the sanitary sacrifice and destruction of the affected colonies, to keep Brazil as an AFB-free country (MAPA, 2006). Because of its economic impact, AFB is a disease with mandatory notification (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2018/).

Chalkbrood is caused by the fungus *Ascosphaera apis*, causing losses to beekeepers mainly in the Northern Hemisphere. Nosemosis is caused by two microsporidia (*Nosema apis* and *Nosema ceranae*). It has intriguing features and its sanitary importance remains poorly understood, although frequently it has been associated with colony losses in Europe (Paxton et al., 2007; Higes et al., 2010). Both, chalkbrood and nosemosis occur in Brazilian apiaries, but they do not seem to cause economic losses. Queen replacement is the main control procedure because the use chemical compounds is prohibited by Brazilian laws.

The objective of this study was to identify by multiplex PCR, the presence of spores of the pathogens *P. larvae*, *A. apis*, *N. ceranae* and *N. apis* in bee products (honey, pollen and royal jelly) registered with the Federal Inspection Service (S.I.F.) of the Brazilian Ministry of Agriculture (MAPA) and available commercially in the state of São Paulo, to contribute to the understanding of the spread of diseases by bee products.

Materials and methods

Seventeen samples of honey, 14 of pollen and 10 of royal jelly were obtained commercially in the state of São Paulo.

For detection of spores of *P. larvae*, *A. apis*, *N. ceranae* and *N. apis* with multiplex PCR, the samples were submitted to the method proposed by Guimarães-Cestaro et al. (2016). Four aliquots (20 mL of honey, 10 g of pollen and 5 g of royal jelly) were analyzed from each commercial sample.

The analysis of pollen samples is summarized in the Figure 1 (Appendix A. Supplementary data). Four samples with 10 g of pollen were transferred to 45 mL of sterile distilled water. The samples were stirred and filtered through Whatmann #1 filter paper in a vacuum. To avoid sample contamination by reflux, the vial was connected by a plastic tube to a Kitasato flask containing a cotton ball with 0.04% peracetic acid. One sample was used for microbiological analysis (Brasil, 2003) and three for molecular analysis (Figure 1, Appendix A. Supplementary data).

The three samples for molecular analysis were centrifuged at 12,500 × *g* for 40 min. The pellet was resuspended in 1 mL of autoclaved distilled water and transferred to a 2 mL microtube, stirred vigorously to homogenize of the suspension and centrifuged at 10,000 × *g* for 20 min. The supernatant was discarded and 100 mg of the pellet was used for DNA extraction with a DNeasy Plant Mini Kit Qiagen® (Qiagen, Hilden, Germany), following manufacturer's instructions and submitted to multiplex PCR analysis according to Puker (2011): an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min.

Microbiological analyses were carried out as parameters for molecular analyses, and were performed following method suggested by the Brazilian government to identify *P. larvae* (Brasil, 2003) using the protocol proposed by Schuch et al. (2001), with modifications: the sample volume used was 300 μL instead of 100 μL, and centrifugation at 12,500 × *g* for 40 min instead 3000 × *g* for 30 min. The samples were spread onto a culture medium consisting of *P. larvae* agar (PLA) with *Bacillus cereus* Select Agar Base (PEMBA) (Oxoid) and Cereus Selective Agar Base (MYP Agar) Mossel (Merck) in triplicate.

Samples of honey and royal jelly were submitted to the procedure described above, except for use of 20 mL of honey and 5 g of royal jelly in 40 mL of sterile distilled water, without filtration (Figure 2, Appendix A. Supplementary data).

All precautions to avoid cross-contamination were taken and controls of extraction and PCR were included. All analyses were carried out with positive and negative controls. Only filter tips were used, associated with all other good laboratory practices.

After PCR, 5 μL of the reaction product was submitted to 2% agarose gel electrophoresis, stained with SYBR SAFE® (Life Technologies, Carlsbad, EUA) in TBE 1× (89 mM Tris base, 89 mM boric acid and 2 mM EDTA) buffer and photographed with E-Gel Imager (Life Technologies®, Carlsbad, EUA). The 100 pb Marker (Invitrogen®, Carlsbad, EUA) was used to determine the fragment size.

The PCR products were purified with the QIAquick Gel Extraction Kit, following the manufacturer's instructions and submitted to sequence reactions with BigDye® Terminator v3.1 Cycle Sequencing Kit and termociclador Veriti® (Applied Biosystems, Foster City, USA). The samples were transferred to ethanol and injected an Applied Biosystems 3130xl automatic sequencer. Sequencing was performed in the Laboratory of Animal Biotechnology of ESALQ/USP, Piracicaba, SP and analyzed in the Molecular Genetics Laboratory of Embrapa Gado de Leite, Juiz de Fora, MG using the BLAST tool available in the GenBank (http://www.ncbi.nlm.nih.gov) to confirm the expected amplicon for each primer pair (average identity equal 99% +/− 0.00 to 1.27 for all pathogens).

Results and discussion

This molecular analysis, simultaneously identified the presence of spores of *Nosema* spp., *A. apis* and *P. larvae* in bee products sold in the state of São Paulo with supervision by the S.I.F. Based in the detection limits of the multiplex PCR (Guimarães-Cestaro et al., 2016), of 41 samples of bee products analyzed, 40 were positive for spores for at least one pathogen (Table 1). *N. ceranae* was detected in 87.80% and *P. larvae* was detected in 85.37% of the samples, while *A. apis* was detected in 73.17% and *N. apis* was present in 26.83%. *N. ceranae* ranged from 85.71% in pollen samples to 94.12% in honey. *P. larvae* ranged from 80% in royal jelly to 92.90% in pollen. *A. apis*

Table 1
Samples of pollen, honey and royal jelly obtained from markets in the state of São Paulo, contaminated with *Nosema ceranae*, *Nosema apis*, *Ascosphaera apis* and *Paenibacillus larvae*.

Matrix	*N. ceranae*	*N. apis*	*A. apis*	*P. larvae*	Simultaneous contamination (samples with more than one pathogen)[a]	Without contamination
Pollen ($n=14$)	12	4	11	13	13	0
Honey ($n=17$)	16	6	14	14	17	0
Royal Jelly ($n=10$)	8	1	5	8	7	1
Total ($n=41$)	36	11	30	35	37	1

[a] The percentage of simultaneous contamination of each product, by sample, can be find in Appendix B. Supplementary data.

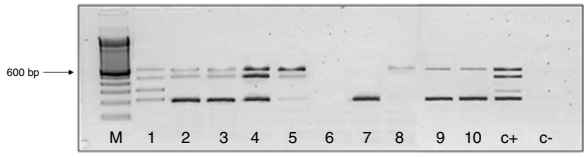

Fig. 3. 2% agarose gel stained with SYBR® Safe of the multiplex PCR products from royal jelly samples (1–10) obtained from markets of the state of São Paulo, Brazil. M, molecular marker 100 pb (Invitrogen®); C+, positive control for *N. ceranae* (218 pb), *N. apis* (321 pb), *A. apis* (485 pb) and *P. larvae* (700 pb); C−, negative control.

occurred in 78.57, 82.35 and 50% of samples of pollen, honey and royal jelly, respectively (Table 1).

Bee products with simultaneous contamination

Considering the total of samples, 90.2% were contaminated with more than one pathogen (Table 1). Of 14 pollen samples analyzed (Fig. 5), 13 were positive for *P. larvae*, 12 (85.71%) for *N. ceranae*, 11 for *A. apis* and four (28.57%) for *N. apis*. Simultaneous contamination was also observed, and two pollen samples were contaminated with all pathogens (more details in Appendix B. Supplementary data). All 17 honey samples were positive for least two of the microorganisms. The microsporidia *N. ceranae* and *N. apis* were simultaneously found in five samples (29.41%) (Fig. 4) (more details in Appendix B. Supplementary data).

In the royal jelly samples analyzed, only one was negative for pathogens and nine were positive for at least one of the pathogens (Fig. 3) (more details in Appendix B. Supplementary data).

Simultaneous contamination by all pathogens occurred in 10% of royal jelly samples (Fig. 3), 17.6% of honey samples (Fig. 4) and 14.3% of pollen samples (Fig. 5) (more details in Appendix B. Supplementary data).

Pollen grains have been reported to be vehicle for transmission of different pathogens afflicting bees, including pathogen spores (Hale and Menapace, 1980; Flores et al., 2005; Higes et al., 2008a; Fries et al., 2013; Pettis et al., 2013), due to the use of contaminated food sources as well as artificial feeding of the colonies with pollen (Gochnauer and Corner, 1974; Hitchcock and Revell, 1963). Bee pollen is used by beekeepers as food for their hives in a period of food shortage; so, transmission of pathogen spores to healthy colonies can occur through contaminated pollen. In addition, due

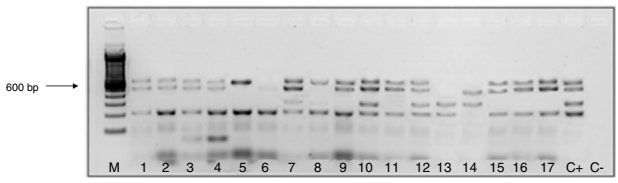

Fig. 4. 2% agarose gel stained with SYBR® Safe of the multiplex PCR products from honey samples (1–17) obtained from markets of the state of São Paulo, Brazil. M, molecular marker 100 pb (Invitrogen®); C+, positive control for *N. ceranae* (218 pb), *N. apis* (321 pb), *A. apis* (485 pb) and *P. larvae* (700 pb); C−, negative control.

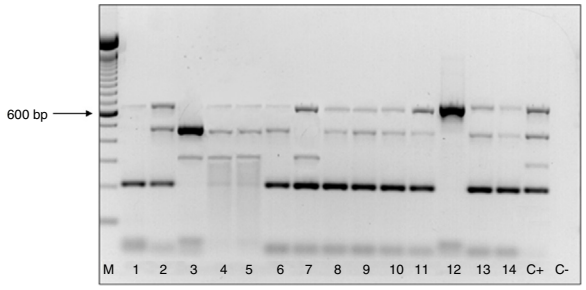

Fig. 5. 2% agarose gel stained with SYBR® Safe of the multiplex PCR products from pollen samples (1–10) obtained from markets of the state of São Paulo, Brazil. M, molecular marker 100 pb (Invitrogen®); C+, positive control for *N. ceranae* (218 pb), *N. apis* (321 pb), *A. apis* (485 pb) and *P. larvae* (700 pb); C−, negative control.

to hygienic behavior, worker bees remove ill brood bees from the colony by throwing them out through the entrance of the hive, where pollen traps are usually attached. Thus, these collectors are potential reservoir of pathogens, including spores, which can remain viable in the environment for decades. Spores viability is ca. 15 years for *A. apis* (Hornitzky, 2010) and from 35 (Haseman, 1961) to 70 years for *P. larvae* (Shimanuki and Knox, 1994, 2000).

Efficiency of multiplex PCR

The multiplex PCR analysis had detection limit of 25 spores for *N. ceranae*, 150 for *A. apis* and eight for *P. larvae* per 20 mL of honey, 10 g of pollen and 5 g of royal jelly, indicting high accuracy in pathogen detection (Guimarães-Cestaro et al., 2016). The centrifugation conditions associated with the volume and preparation procedures of the bee products analyzed may have optimized the spore recovery, resulting in a high spore concentration with a large number of spores in the pellets submitted to DNA extraction and subsequent multiplex PCR analyses.

Comparison of extracting DNA of microorganisms directly from bee products (Figures 1 and 2, Appendix A. Supplementary data) with the conventional PCR approach using primers of previously known specificity (Murray et al., 2005; Piccini et al., 2002; Martín-Hernández et al., 2007) indicates that this technique is an important alternative for use in laboratories to identify pathogens in bee products

According to the OIE (2016), some methods to identify *P. larvae* need culture of bacteria in a microbiological environment, whereas others can be conducted directly from material collected in colonies of apiaries. In this study, commercial bee products (honey, pollen and royal jelly) were analyzed considering the possibility of pathogen dispersion by this route, since in Brazil there is no systematic program regulated by the federal government to monitor the health of commercial hives, even though the country is contiguous to neighbors where AFB occurs (representing a constant threat). Among other factors, the main bottlenecks for the implementation of such programs are the shortage of laboratories for this diagnosis (there is only one official laboratory), the absence of

rapid and sensitive official detection techniques (other than microbiological ones), and the lack of beekeeper registration and animal traffic control, which are important for agricultural defense system. In addition, the continental size of Brazil poses show the difficulties of sample collections *in loco* and the volume of samples to be analyzed by a diagnosis technique that takes more than a week to produce results.

The samples used in this study were restricted to the state of São Paulo, the largest honey exporter (http://brazilletsbee.com.br/inteligencia_comercial_abemel_abril_2015.pdf), although this honey may come from other Brazilian regions (Fachini et al., 2013).

Although sophisticated molecular techniques such as real-time PCR have advantages in speed, sensitivity and practicality compared to conventional PCR, the latter has a reduced financial cost compared to the former, besides higher sensitivity and anticipation of diagnosis in at least one week when compared to microbiological approaches. This higher sensitivity of PCR in relation to the microbiological technique for identification pathogens is discussed by Graaf et al. (2006), considering the possibility of detection of genetic material of non-viable cells of *P. larvae*, suggesting that may be detected dead or non-germinating spores, both unimportant for the disease, but important for pathogen detection. Our results corroborate this hypothesis, with the presence of *P. larvae* in 85.37% of the 41 samples analyzed. Only four pollen samples showed bacterial colonies catalase negative indicating the presence of *P. larvae* (Brasil, 2003). The positive controls have growth characteristic of the bacterium and in the negative controls there was no growth. Thus, the PCR results show that the sensitivity of this technique is higher in the presence of spores of the pathogen, with results obtained in one day and without prior culture or inconsistency of the microbiological results. Inconsistency between microbiological and real-time PCR tests for *P. larvae* identification was reported by Chagas et al. (2012) for bacterial colony without all confirmatory tests as recommended by official technique (Brasil, 2003), showing that the later has low sensitivity. The protocol of the Brazilian official technique (Brasil, 2003) is based on Schuch et al. (2001), but which preceded the scientific and technological advances in this matter (Graaf et al., 2006).

Our microbiological analyzes were carried out as a comparative parameter for sensitivity with the molecular technique using PLA agar medium – *P. larvae*, with *B. cereus* agar Oxoid CM 617 or with Merck Agar MYP in triplicate (Figures 1 and 2, Appendix A. Supplementary data).

The revision of the *P. larvae* subspecies into a single species and four genotypes, ERIC I to ERIC IV, occurred later (Genersch et al., 2006). This may explain the absence of some characteristics suggested by Schuch et al. (2001), such as the negative catalase cultures. Genersch et al. (2006) and OIE (2016) reported that isolates of *P. larvae* can be catalase-negative (ERIC I and ERIC II) or weak-delayed positive (ERIC III and ERIC IV), and the isolate used here as positive control (ATCC 9545) is ERIC I (Genersch et al., 2006).

The delays in obtaining results of microbiological culture techniques, associated with the fastidious characteristic (with precise nutritional and environmental requirements) of *P. larvae* and the need for rapid diagnosis of the pathogen, means the multiplex PCR method has a significant advantage in the rapid time for detection as well as in the number of samples analyzed concomitantly, since it detects multiple DNA sequences of different pathogens in a single reaction, simplifying the workflow and reducing processing time (Bilgiç et al., 2013).

Among few studies reporting the detection of pathogens in bee's products with PCR, without previous microbiological culture, few report the detection threshold. Identification of *P. larvae* in honey artificially contaminated with 170 spores/mL, following DNA dilution to 10^{-3}, was performed by Piccini et al. (2002), whereas Chagas et al. (2010, 2012) suggested the analysis of honey and pollen samples with real-time PCR to identify the bacteria, but from the growth colonies in culture medium.

The association of microbiological and molecular techniques is often proposed for identification of *P. larvae* in honey (Antúnez et al., 2004; Graaf et al., 2013), pollen and royal jelly (Graaf et al., 2013). In addition, adult bees and wax debris can also be used for the diagnosis of *P. larvae* (Ritter, 2003; Graaf et al., 2013).

Nosema ceranae *and* N. apis

Our results showed that *N. ceranae* was the most prevalent pathogen, detected in 80% of royal jelly, 94.1% of honey and 85.7% of pollen samples, while *N. apis* was detected in 10% of royal jelly, 35% of honey and 28.6% of pollen samples (Table 1).

N. ceranae was identified in *Apis cerana* (Fries et al., 1996) and has rapid dissemination in *A. mellifera*, occurring in five continents (Higes et al., 2006; Klee et al., 2007; Paxton et al., 2007; Fries, 2010). This dispersion may be due to transit of bees, either naturally transported legally or illegally, without accurate sanitary screening, in addition to trade in bee products contaminated with the microsporidium or by use contaminated wax. The importation of queens without health control also is a potential risk for pathogen dispersion.

In a long-term laboratory cage study, Williams et al. (2014) demonstrated that parasitism by *Nosema*, in particular by the invasive *N. ceranae* compared to the historic *N. apis*, increased honey bee worker mortality. They also observed higher spore intensity in honey bees parasitized by *N. ceranae* compared to *N. apis*, and a numerical response in spore production during co-infection; this is likely important to inter-host horizontal parasite transmission that relies on ingestion of spores. Recently, McGowan et al. (2016) showed that although the median infective dose of *N. ceranae* was 149 spores per bee, the minimum dose capable of causing a detectable infection was very low (1.28 spores), suggesting that differences in reproduction and intra-host competition may explain apparent heterogeneous exclusion of the historic parasite *N. apis* (Williams et al., 2014). Therefore, even only a few spores in bee products could spread the pathogen and be one of the relevant forms of dispersion of the disease.

Teixeira et al. (2013), stated that simultaneous contamination with two species of *Nosema* in same colony is rare, since from 637 samples collected between 2009 and 2012, in 47 municipalities in 10 different Brazilian states, 0.63% were contaminated simultaneously with *N. apis* and *N. ceranae*, the former found in 0.31% of the samples.

Although this pathogen is present in several parts of the world (Klee et al., 2007; Paxton et al., 2007; Fries, 2010), there are no official techniques for their detection in bee's products indicated by the World Organization for Animal Health in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2016). Another widely used as reference is the *Beebook*, which is a collection of standard protocols for research on bee's pathogens, where detection of *Nosema* spp. (Fries et al., 2013) and of *A. apis* (Jensen et al., 2013) in bee products are lacking.

Besides the PCR used here, Giersch et al. (2009) indicate centrifugation at 3000 × *g* for 45 min before PCR for detect *Nosema* spp. in honey. Although the technique here presented higher centrifugation time and speed, it is noteworthy that it is performed in order to simultaneously detect the three pathogens analyzed.

Spores of *N. ceranae* are found not only in midgut cells, but also in hypopharyngeal glands (Chen and Huang, 2010) and in samples of royal jelly from China (Cox-Foster et al., 2007). Our findings show that honey may be a dispersing vehicle of *N. ceranae*. Giersch et al. (2009) found this microsporidium in honey samples from Australia and some other countries and Hamiduzzaman et al. (2010) report

detection of *Nosema* spp. in honey with molecular analyzes. Botias et al. (2012) stated a positive correlation between increase in *N. ceranae* infection and its occurrence in honey.

Differential immune activation may be involved if the higher dose triggered a stronger larval immune response that resulted in fewer adult spores but imposed a cost, reducing lifespan (Eiri et al., 2015).

Paenibacillus larvae

In relation to occurrence of *P. larvae* in honey, the first molecular protocol that addresses the sensitivity of the technique, was provided by Bakonyi et al. (2003), where the sensitivity of PCR was evaluated by dilutions of DNA isolated directly from the bacterial colonies (thus requiring the use of culture media) and not directly from bee's products. Although some spores detected by the molecular technique may be dead, it is a more practical, quick and lower cost analysis, indicating the presence of the pathogen and ensuring rapid decision-making by the Official Veterinary Agencies.

In artificially contaminated honey, Bassi et al. (2010) compared microbiological and molecular methods for the diagnosis of *P. larvae*. Both methods were sensitive to values greater than 8 CFU/mL of contaminated honey. The authors claim that PCR provides a fast rate to obtain result (<24 h) when compared to the microbiological method (eight days). In Brazil there are similar problem, considering that the official microbiological technique (Brasil, 2003) adopted by the Ministry of Agriculture, to detect the pathogen in honey can take up to nine days. Our proposal contemplates the adaptation of multiplex PCR technique to identify different pathogens and their spores that affect bees with the use of conventional PCR technique.

In Brazil, the American foulbrood is considered an exotic disease, and one outbreak was officially detected in the state of Paraná at 2006 (MAPA, 2006) following its eradication. In this outbreak, samples were collected for analysis in 14 apiaries with 69 colonies contaminated following the burning of all 246 hives, without the identification of the origin of primary focus (E. Kruger, personal communication, SFA-PR/MAPA).

For detection of *P. larvae* bacteria in honey, the OIE (2016) indicates microbiological analysis with honey sample diluted in an equal volume of PBS and centrifuged at 6000 × *g* for 40 min. However, in the Brazilian official technique (Brasil, 2003) the centrifugation is 3000 × *g* for 30 min. In the protocol proposed in this research samples were centrifuged at 12,500 × *g* for 40 min in order to detect not only *P. larvae*, but also *Nosema* spp. and *A. apis*, because use of samples with mild centrifugation have spores in the supernatant, and the aliquot analyzed was the pellet.

In pollen samples, we obtained a successful filtration of 10 g pollen with the aid of a vacuum pump. In the technique recommended by the OIE (2016), however, it is suggested to dilute 1 g of pollen in 10 mL of sterile distilled water and simple filtration with Whatman-1 filter paper. However the use of a vacuum pump allowed rapid filtration of greater pollen samples (10 g), resulting in high accuracy, because it increases the possibility of detection due to greater amount of sample analyzed as well as of smaller number of spores retained on filter paper, due to the use of vacuum pump. It is noteworthy that the filter paper used was Whatman-1 with 11 μm pores, in contrast with Whatman-2 indicated by Brazilian official technique with 8 μm pores, retaining more spores associated with pollen (Higes et al., 2008b; Fries et al., 2013; Pettis et al., 2013).

For molecular detection of the bacterium in royal jelly, the OIE (2016) indicates the same methodology used for larvae, adult and wax, but samples of this product can be submitted to PCR after centrifugation of 6000 × *g* for 30 min using 1–5 μL of the supernatant as DNA template in a 50 μL PCR reaction. Our spore recovery tests, artificially inoculated in sterile royal jelly, showed that the speed of 12,500 × *g* for 40 min was better, with greater recovery of the pathogen in few amount (5 g) of royal jelly.

The contamination of 80% of the royal jelly samples with *P. larvae* and 50% with *A. apis* suggest that royal jelly has not antibiotic effect against spores of these two pathogens. The peptide royalisin found in the royal jelly has been reported as a potent antibiotic against gram-positive bacteria (Fujiwara et al., 1990), *P. larvae* and fungus (Bíliková et al., 2001), avoiding growth of bacteria and fungi (Fujiwara et al., 1990; Bíliková et al., 2001; Fontana et al., 2004). However, the bee's products here analyzed have spores and not by the vegetative forms.

It is expected that bee's products such as honey and pollen from diseased hives from countries with occurrence of American foulbrood have *P. larvae* spores. Honey extracted from colonies infected with 25 million spores per gram of bee pollen has about 4.5 million spores per gram (Gochnauer, 1981). However, the presence of *P. larvae* spores in honey formally marketed with Federal registration in the Brazil, where the disease is not recognized is not expected. Our molecular analyzes are not quantitative, but show the presence of the pathogen in the products, which suggests both the possibility of the disease in Brazilian apiary suppliers of such products (we must investigate if clinical signs are present or if spores are present, even in the absence of clinical signs), as well as that there may be commercialization of contaminated products imported. In the case of royal jelly, pollen analyses indicate the second hypothesis is more suitable because pollen grains in this bee's product were from many exotic plants rarely cultivated or not cultivated in Brazil (unpublished data).

In the samples of honey and bee pollen contaminated, pollen analyses showed the presence only of native plants (unpublished data). Thus the contamination may be due mixtures of several samples in the warehouses. This fact may have impaired the evaluation of the original pollen spectrum, with predominance of the pollen types of native plants mixed. As the production of royal jelly in Brazil became economically unviable after the importation of Chinese royal jelly in 1990 decade, the analyzed samples are not mixed with Brazilian royal jelly.

The dispersion of spores by imported bee's product without sanitary evaluation is the principal concern associated with routes of contamination: (i) food supplementation with infected products during periods of food resources decrease; (ii) use of contaminated royal jelly (purchased at low prices in trade from China) for queen production, (iii) handling of imported contaminated product and posterior use of tools in healthy colonies, (iv) bees' access to debris of contaminated products discarded in the vicinity of colonies (e.g. pollen dryers equipment, where pollen from different places are dehydrated), and (v) use of contaminated wax. Current management practices such as the transfer of combs between colonies, as well as the use of the same tools and trophallaxis among bees could spread contamination too.

Future studies should also quantify pathogen levels, besides identify specific haplotypes to estimate potential risk of spread.

Actions taken

As an immediate consequence of the results obtained, MAPA was officially notified for the presence of *P. larvae* in products obtained in the marketed in state of São Paulo, as determined for OIE. This shows the importance of our research for aid governmental guidelines, aiming the protection of national beekeeping, with real risk of dispersion of pathogens with trade of bee's products. In addition, we demonstrate the fragility of the inspection offices for the control of imported products, without official supervision and, consequently, without the sanitary screening required by law, as supported by pollen analyses of the royal jelly samples, indicating

presence of pollen grains from Europe and Asia plants, which are contaminated with pathogens that affect *A. mellifera* and that these products onset to be marketed as Brazilian products.

It is clear that bee pathogens and pests are able to contaminate bee products that are traded globally. There are standards for global trade in live bees and bee products. Even with these international guidelines, pests and pathogens continue to move around the globe since many countries lack the resources and expertise to inspect and certify bee products as disease-free (Pettis, J., personal communication). This seems to happen in Brazil as well.

Conclusions

A single PCR analysis showed the presence of spores of *N. ceranae*, *N. apis*, *A. apis* and *P. larvae* on samples of honey, pollen and royal jelly marketed in the state of São Paulo, with registration in S.I.F.

These results may be useful as subsidies for governmental regulations in the area of sanitary control of exotic diseases to prevent illegal imports or dispersion of pathogens mainly increasing the sanitary control of bee's products, since local and international trade and transference of colonies between regions may play an important role in the pathogens dispersion.

Our results indicate the urgent need for research to identify the *P. larvae* strains found in contaminated products. The occurrence of *A. apis* and *Nosema* spp. spores cannot be neglected, although these pathogens cause lower damage to beekeeping than *P. larvae*.

Overall, our findings indicate the necessity to investigate the origin of contaminated bee's products with use of molecular approaches.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rbe.2018.04.001.

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