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A PCR method of detecting American Foulbrood (*Paenibacillus larvae*) in winter beehive wax debris.

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Abstract

The objective of this work was to create a fast and sensitive method of detecting *Paenibacillus larvae* from beehive debris based on PCR that does not require long-lasting cultivation steps. Various methods of extracting spores from beehive debris were compared: the original method of extraction of spores into toluene, and alternative spore extraction methods into Tween 80, into water, into isopropanol and into 95% ethanol. Isolation of DNA from various spore extractions was evaluated too. Best results were provided by isolation of DNA using the QIAamp DNA Mini Kit, without heat treatment. DNA of spores were detected by PCR from 0.25 g of beeswax debris, with the detected titer of 10^5 in 1 g according to the cultivation tests.

Keywords: Honeybee, *Paenibacillus larvae*, AFB, American foulbrood, hive debris

Introduction

As other animals the honey bee (*Apis mellifera* L.) is inevitably attacked by infectious pathogens which can result in loss of the whole colony. American foulbrood (AFB) is one of the pathogens which is a serious threat and cause for significant decline in honeybees (Hansen and Brodsgaard, 1999). AFB is an infectious bee disease that is caused by a spore forming rod-shaped bacteria *Paenibacillus larvae* which has spread worldwide (de Graaf et al., 2006b; Genersch et al., 2006; Riessberger-Galle et al., 2001). This cosmopolitan and highly infectious disease affects larval and pupal stages during the honey bee development. Dead larvae decompose to a glue-like colloid liquid, producing a specific smell. The bacteria creates spores covered by 7 protective layers which enable it a life-span of at least 30-50 years (Bakhiet and Stahly, 1985). Later the liquid dries and it remains at the bottom and on the walls of cells, where it creates a typical scale of a brown-black color. Honeybees try to remove this scale from cells and spread spores to other larvae. The disease is so infectious that it can destroy the infected honey-bee colony within a few months or years (Hansen and Brodsgaard, 1999).

Regarding the importance of early detection and due to the threat of high losses, developing of a fast and reliable method for early detection of infectious spores seems of high importance. Traditional methods are reliable but rather slow simply because they are based on biochemical and morphological / physiological identification of cultivated isolates (Reynaldi and Alippi, 2006). Other methods are based on specific reactions of bacteriophage BL-2, which attacks only *Paenibacillus larvae,* that can be used for fast diagnosis of supposed isolates (Stahly et al., 1999). Nevertheless, this method also requires relatively long cultivation. The development of methods of detection of *Paenibacillus larvae* based on PCR and nested PCR (Lauro et al., 2003) has been described earlier (de Graaf et al., 2006a; Govan

et al., 1999). Such methods focused on the detection of AFB in brood, in foulbrood scales and in honey (Alippi et al., 2004; Bakonyi et al., 2003; Dobbelaere et al., 2001).

The main objective in the present study was to verify the sensitivity and reliability of methods based on PCR for the detection of AFB from bee-hive debris and to compare it with cultivation tests. Various methods of spore extraction from debris were compared: the original method of spore extraction into toluene (Titera and Haklova, 2003), a method using Tween-80 (Bzdil, 2007), a method of spore extraction into water, isopropanol or 95% ethanol. Individual procedures of extraction were performed either by a cold method or by a method with heat treatment of the sample. For the purpose of decoating DNA from spores methods using NaOH/SDS (Vary, 1973), the combination of Lysozym, Proteinase K (d'Alessandro et al., 2007) and TritonX and a method without any special lysis of spores were compared. The reliability and sensitivity of PCR tests was compared with the classic cultivation test (Titera and Haklova, 2003).

Materials and Methods

Extraction of spores from debris

Toluene method of extraction: The quantity of 1 g of debris was dissolved in 9 ml of toluene for the purpose of the test and the final blend was shaken at room temperature. An hour later the portion of wax dissolved in toluene was transferred into a new tube and 2 ml of distilled water were added. The mixture was heated at 90^{o}C for 5 minutes and vigorously mixed (Titera and Haklova, 2003). After cooling, 200 µl of the water phase was used for inoculation MYPGP agar and 100 µl of the water phase was used for the consequent PCR test.

Tween method of extraction: The amount of 1 g of debris was added into a tube with 8.5 ml of sterile distilled water and the final blend was completed with 0.5 ml of preheated (at 70^{o}C) Tween 80. The final blend was shaken and heated in water at 70°C. Consequently the blend

was cooled and 3 ml were taken on water phase, transferred into the same volume of distilled water then further 1 ml was taken for the release of DNA from spores. The final mix was heated at 90^{o}C for 10 minutes.

Spore extraction into isopropanol and ethanol: The quantity of 1 g of debris was added for the test to 9 ml of isopropanol or to 9 ml of 96% ethanol and the mixture was shaken at room temperature. A volume of 100 µl was taken after an hour for isolation of DNA for the PCR test (cold method). The mixture was heated at 70^{o}C for a period of 5 minutes and vigorously mixed. After cooling, another 100 µl of the water phase was added (warm method) for the isolation of DNA for the PCR test.

Extraction of spores into water: The quantities of 0.2 g, 0.5 g and 1 g of debris were added to 5 ml of distilled water and the mixture was shaken at room temperature. After an hour 100 µl were taken for the isolation of DNA for PCR test (cold method) and the remaining mixture was heated to 70^{o}C for a period of 5 minutes and vigorously mixed. After cooling of the mixture another 100 µl of water phase (warm method) were used for isolation of DNA and consequent PCR tests.

Lysis of bacterial spores

Enzymatic lysis by the Lysozym: The quantity of 10 µl of TE buffer containing 20 mM Tris-HCl (pH 8.0) was added to each sample, 2 mM EDTA, 0.5% (w/v) Triton-X, 20 mg Lysozyme for 1 ml and the resulting mixture was incubated at 37°C for 1 hour. After the incubation 5 µl proteinase K were added and the lysate was incubated at 56°C for another 30 minutes. Consequently 200 µl of buffer AL (Qiagen) was added and next incubation was performed for at 56°C 30 minutes. Bacterial DNA was isolated by using the QIAamp DNA Mini Kit (Qiagen) according to instructions.

Method NaOH/SDS: A total amount of 1 ml of sample (after extraction of spores) containing 0.1M NaOH, 0.1M NaCl (pH 10.8), 1% SDS (w/v) and 0.1% DTT was incubated at 70°C (Vary, 1973) for 30 minutes. Bacterial DNA was isolated with QIAamp DNA Mini Kit according to the manufacturer's instructions.

Cultivation AFB test

Fifty samples of beeswax debris were obtained from apiaries in the territory of the Czech Republic. Each sample contained a mixture of debris originating from a single apiary (approx. 5-30 bee colonies) and weighed approximately 100 g. The quantity of 1 g of beeswax debris was dissolved in 10 ml of toluene. The mixture was shaken for about an hour. Then the total of 2 ml of the dissolved material was transferred into 6 ml of salt solution (120 mM NaCl). The mixture was shaken and the total of 200 µl was used for inoculation on MYPGP agar with nalidix acid (30 mg/l) depressing growth of microbes. All elements (except glucose) were dissolved in water and sterilized at 121°C for 20 minutes. Glucose was sterilized by filtration and added into the partly cooled medium. All samples were cultivated in triplicates at 37°C for 7 days.

Primer design & PCR

Desalted primers were used to amplify 451 bp fragment of the AFB gene (PL1-f: GCT CTG TTG CCA AGG AAG AA and PL1-r: AGG CGG AAT GCT TAC TGT GT) (Bakonyi et al., 2003). The PCR reaction contained PCR buffer, 1.75 mM $MgCl_2$, 1 µM of each primer, a further 1.5% formamide, 2.5% DMSO and 1 M Betaine, 2µl of template and 2U HotStart Taq polymerase. Thermal cycling included following steps: 15 min at 95°C, 35 cycles consisting of denaturating at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min.

Samples of the amplicons were electrophoresed in 1.0% agarose gel. Approximate product size was determined using the 100-bp molecular size ladder.

Results

Out of 50 samples tested for AFB six were positive using cultivation tests. Positivity was confirmed by PCR tests. Comparison of spore extraction into solvent: toluene, Tween 80 (data not shown), isopropanol, ethanol and water are shown in Fig.1. Extraction of AFB spores into water showed the highest efficiency (Fig.1, line 3-5), while extraction into toluene (common in cultivation tests) gave consistent results (Fig.1, line 7-8). The extraction into isopropanol and ethanol showed the average value of efficiency (Fig.1, line 1-2). After extracting spores into solvent, DNA was decoated. DNA that was decoated from spores with NaOH/SDS showed worse results than lysis of AFB spores by common procedure using QIAamp DNA Mini Kit method, while Lysozym and TritonX had improving effect. Heat treatment upon lysis and decoating spores (70°C, 15 minutes) caused a decrease in DNA yield or its partial degradation. Best result was provided by isolation of DNA from spores of AFB in beeswax debris by using the QIAamp DNA Mini Kit, by cold method and without the use of other materials, which decompose the wall of spores. Spores were detected by PCR in the weight of 0.25 g of beeswax debris in the titer of 10^5 in 1g (based on the cultivation test).

The sensitivity of PCR was compared with the cultivation technique. AFB was found in 6 samples of the total number of 50 samples using cultivation tests. The titer of the amount of spores *Paenibacillus larvae* was set in the range of 10^5 (2 samples), 10^3 (2 samples) and 10^2 (2 samples) using cultivation tests. All positive samples, found in cultivation tests, were confirmed by PCR.

Comparison of types of extractions: Six samples of AFB positive with titer of 10^5 (by using the cultivation tests) and 1 sample of AFB negative debris were selected for the purpose

of comparison of spore extraction from debris into solvents. Toluene (used for cultivation tests), isopropanol, ethanol (96%), water + Tween80 and water were selected as the solvents. Best result was achieved by the extraction into water (Fig.1, line 5), a slightly lower efficiency was achieved when using extraction into isopropanol and ethanol. Extraction into water with Tween 80 did not show any differences compared with water alone.

Comparison of heat treatment of samples: Beeswax debris with the titers 10^5 and 10^2 were heat treated after shaking in toluene and water at 90°C for a period of 15 minutes. Heat treated samples had significantly worse or no results in comparison with the control without heat treatment.

Discussion

Reliable detection of AFB plays an increasingly important role in preventing loss of bee colonies and has become a common routine. Even a small amount of spores suffices to infect sensitive bee brood. In a short time an outbreak of the disease can result with fatal consequences for a bee colony or often for a whole commercial farm. Such outbreak can spread to distant places. The sooner the focus of infection is located, the smaller the costs for its disposal. From this point of view the methods of detection, prevention and timely identification of this disease are important tools for reducing AFB.

PCR is a reliable, fast and widely-used method in microbiological diagnostics and the testing of DNA from pathogens is an alternative to the classic cultivation tests on agar. Many types of samples can be taken as a source of infectious material for testing the AFB. Adult bees (Lindstrom et al., 2008), larvae of brood and pupae are very efficient as AFB spreads there (Bakonyi et al., 2003), however, it is difficult and time-consuming to find well trained staff, who has to search for clinical symptoms and take sterile samples from the suspicious bee colonies within the short summer period. It is possible to take honey (Crailsheim and

Riessberger-Galle, 2001), but the concentration of spores in honey is much lower and the difficulty involved in sample taking is also a disadvantage. Analysis of such samples may provide false negative results. A sensible possibility is sampling of beeswax debris from the bottom of beehives for a given period of time (e.g. one month) and its analysis during winter. Debris consists of petty wax elements, which are created during the winter period during the uncapping of food store combs. They fall to the bottom of a beehive where it is possible to collect them on a mat placed at the bottom of the beehive all year long and especially during the winter. Debris is not carried out of the hive by the bees during winter. The presence and number of mites *Varroa destructor* and evidence of AFB in the mixed sample are determined in the laboratory using the cultivation test (Titera and Haklova, 2003). The detection of AFB in debris collected during winter is a reliable, simple and non-invasive method of obtaining biological samples from apiaries during the quiet part of the year, because debris collected on the bottom of the beehive gives indication of the health state of the bee colony in the observed period. Debris can be transported without special requirements (brood and bee samples should be cooled or frozen). The method requires no long-lasting cultivation step, thus it is rapid and sensitive. The analysis presented in this paper can detect sick bee colonies or whole apiaries during the winter and may help to avoid a clinical outbreak of the disease and its spreading during summer.

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257
258

Figure legend

FIG. 1. Gel electrophoresis of PCR-amplified fragments of DNA *Paenibacillus larvae*. DNA amplicon 451 bp; Lines: M, molecular size marker (100-bp ladder); 1, extraction into isopropanol, 1g beehive debris; 2, extraction into ethanol, 1g beehive debris; 3, extraction into water, cold method, 1g beehive debris, titer 10^2; 4, extraction into water, cold method, 1g beehive debris, titer 10^2; 5, extraction into water, cold method 1g beehive debris, titer 10^2; 6, negative control; 7-8, extraction into toluene, 1g beehive debris, treatment by temperature 70°C;.