

Full Article

Development of PCR method for diagnosing of honey bee American Foulbrood disease

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Received 26 Feb 2011; accepted 31 May 2011

ABSTRACT

American foulbrood (AFB) disease is caused by the sporeforming bacterium *Paenibacillus larvae larvae*. Traditional diagnosis is based on culture technique is time and laboratory work consuming. In this study with standard strain, PCR was developed by specific primers and PCR products were electrophoresed on 0.8 % agarose gel. The PCR primers were selected on the basis of the 16S rRNA gene and amplify a 700-bp amplicon. Detection limits were determined for suspensions of bacteria and spores and also honey and larvae experimentally contaminated. The lowest number of bacteria and spores that were able to detect were respectively 28, 33, 330 and 243 cfu /ml. This PCR technique can be used to identification of the presence of *Paenibacillus larvae larvae* spores in honey samples, brood samples or on the colonies that grow on medium.

Keywords: Honey bee, AFB, PCR, *Paenibacillus Larvae Larvae*, Spore

INTRODUCTION

American foulbrood (AFB) is one of the pathogens which is a serious threat and cause for significant decline in population (Hansen & Brodsgaard 1999). The causative agent of AFB is Gram positive, spore forming, rod-shaped bacteria *Paenibacillus larvae larvae* which is spread worldwide (Heyndrickx *et al* 1996). This infectious disease affects larval stages during the honeybee development. Dead larvae decompose to a glue-like colloid liquid, producing a specific smell. The bacteria create spores covered by

seven protective layers which enable it a life-span of at least 30–50 years (Bakhiet & Stahly 1985). The infection can remain subclinical for several years (Fries *et al* 2006). The disease spreads when spores are carried on drifting bees, hive parts, beekeepers' clothes, and contaminated pollen or honey (Alippi *et al* 2002). The development of a disease outbreak is thought to depend on the resistance of the bees, the infection load, as well as on the virulence of the infective strain (Pentikainen *et al* 2008). The inspection of honey, larvae and other hive materials in order to detect spores of the pathogen may be of value in tracing AFB outbreaks (Govan *et al* 1999). Culture and chemical tests are very time-consuming and expensive. The

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conventional laboratory diagnosis to obtain a pure bacterial culture is prevented by the slow growth rate of *P. larvae larvae* and the presence of numerous other bacteria. The main objective in the present study was to apply the polymerase chain reaction (PCR) on the 16S RNA gene of *Paenibacillus larvae larvae* (Piccini et al 2002) in pure cultures, spores in experimentally contaminated honey and larvae samples and to assess the sensitivity and reliability of this method by plate count.

MATERIALS AND METHODS

Bacterial strain and culture conditions. Standard strain was obtained from Pasteur Institute of France CIP 104618. Spores were obtained by inoculating one colony of standard strain in 10 ml of sterile distilled water and incubating 72 h at 37 °C. After incubation the presence of spores was confirmed by light microscopy (Piccini et al 2002). One ml of bacterial or spores suspension, inoculated to 5 plates (200 µl per each plate) of MYPGP agar (Mueller-Hinton broth 10 g, yeast extract 15 g, K₂PO₄ 3 g, glucose 2 g, Napyruvate 1 g, agar 20 g and distilled water 1000 ml, supplemented with 9 µg/ml of nalidixic acid, 20 µg/ml Pipemidic acid, 16.8 µg/ml Amphotricin B). Inoculated plates, were incubated at 34 – 37 °C for 2 – 4 days in an atmosphere of 5 – 10 % CO₂ [(Antunez et al 2004) and (Alessandro et al 2007) and (OIE Manual 2010)].

DNA extraction. To obtain DNA from bacterial cultures, a colony of *Paenibacillus larvae larvae* isolate suspended in 50 µl of distilled water and heated at 95 °C for 15 min. After centrifugation at 5000 g for 5 min, the supernatant was used as DNA template for each reaction. One microlitre was used for PCR assays (Govan et al 1999) and (Neuendorf et al 2004).

To obtain DNA from spores, spore suspensions were centrifuged at 6000 g for 30 min to obtain a pellet. Then, the pellets were treated by the following protocol. Pellets were incubated in buffer containing 0.1 M NaOH; 0.1 M NaCl (pH 10.8); 1% SDS (w/v)

and 0.1 M DTT for 30 min at 70°C in a static water bath, mixing every 10 min (Vary 1973) and (Paidhungat & Setlow 2000). After incubations, the samples were washed with PBS by centrifugation and digested with lysozyme (1.5 g l⁻¹ final concentration in TE, for 1 h at 37 °C in a shaking bath). Then, SDS and proteinase K were added (1% w/v and 0.2 g l⁻¹, final concentrations respectively) and incubated in a shaking water bath for 1 h at 50 °C. A salting-out procedure (Bickley & Owen 1995) was performed to precipitate proteins that could interfere in further steps. Briefly, digestions were mixed with cold ammonium acetate to a final concentration of 2.5 M, placed on ice bath for 10 min and centrifuged for 5 min at 12000g. The supernatants were mixed with two volumes of cold 100% ethanol and stored at 20°C overnight for precipitation of nucleic acids. All samples were re-suspended in 50 µl TE and used as template DNA for PCR (Alessandro et al 2007).

PCR. Specific primers were selected from a region of 16S rRNA gene deposited in the available databases that was not homologous to other closely related bacteria (accession number AY030079). These primers were described by Piccini et al. (2002). Primers used were P₁₅: 5'-CGAGCGGACCTTGTGTTTCC-3', and P₁₄: 5'-TCAGTTATAGGCCAGAAAGC-3'. The expected amplification fragment size was about 700 bp. PCR was performed in a final volume of 25 µl containing: 2.5 µl of 10 x PCR buffer, 0.5 µl of 10 mM dNTP mix, 1 U of Taq DNA polymerase, 1 µl of 10 µM of each primer and 2 µl of 25 mM MgCl₂. All PCR materials were from Fermentas Company. The cycle initially was used a 95 °C (1 min); 30 cycles of 93 °C (1 min), 55 °C (30 s), 72 °C (1 min); and a final cycle of 72 °C (5 min). PCR products were visualized by electrophoresis in 0.8% (w/v) agarose gels stained with ethidium bromide (Piccini et al 2002) and (Sam brook et al 1989).

Specificity of PCR primers. To reveal the specificity of the PCR, reactions were tested with the following bacterial strains of various species of gram positive and gram negative.

Paenibacillus larvae standard - *Paenibacillus larvae* larvae wild - *Bacillus cereus* - *Bacillus subtilis* - *Listeria monocytogenes* - *Staphylococcus epidermidis* - *Escherichia coli* - *Salmonella enteritidis* - *Citrobacter freundii* - *Enterobacter sakazakii* - *Klebsiella pneumonia* - *Morganella morganii*.

Sensitivity of the PCR assay. The serial bacterial and spore dilutions of 10^{-1} to 10^{-6} were prepared and 1ml of each dilution was used directly for DNA extraction and PCR, another 1ml for culture and by plate count. Also other 1 ml of each spore dilutions were added to 20 grams of honey and 2 healthy larvae, then DNA extraction and PCR was performed.

RESULTS

The test of PCR specificity showed that 10 out of 12 bacterial strains were negative. Two positive reactions (L_2 , $L_3 = 700$ bp) are related to *Paenibacillus larvae* larvae standard strain and wild strain (Figure 1).

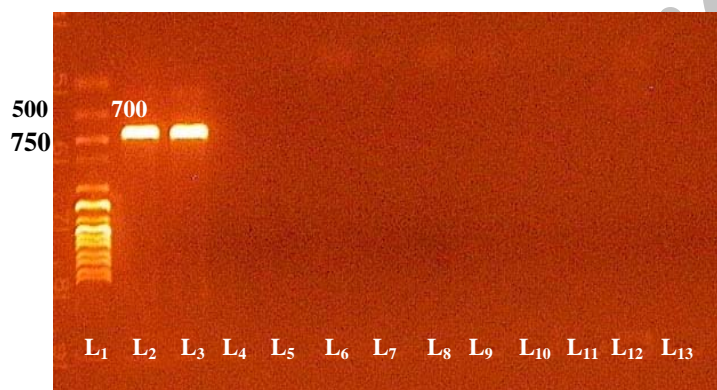


Figure 1. L₁: Ladder 1 Kb - L₂: *Paenibacillus larvae* standard - L₃: *Paenibacillus larvae* larvae wild - L₄: *Bacillus cereus* - L₅: *Bacillus subtilis* - L₆: *Listeria monocytogenes* - L₇: *Staphylococcus epidermidis* - L₈: *Escherichia coli* - L₉: *Salmonella enteritidis* - L₁₀: *Citrobacter freundii* - L₁₁: *Enterobacter sakazakii* - L₁₂: *Klebsiella pneumonia* - L₁₃: *Morganella morganii*

The sensitivity of PCR for bacterial and spore form of *Paenibacillus larvae* larvae were determined, respectively 28 and 33 cfu /ml at 10^{-4} dilutions (Figures 2 and 3). The sensitivity of PCR in experimentally contaminated honey and larvae were determined, 330 and 243 cfu /ml at 10^{-3} dilutions (Figures 4 and 5).

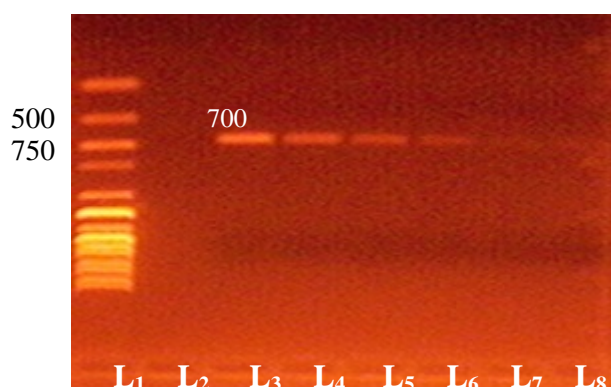


Figure 2. Sensitivity of PCR in bacterium
L₁: molecular size marker (1-kbp ladder); L₂: negative control; L₃: titer 10^{-1} ; L₄: titer 10^{-2} ; L₅: titer 10^{-3} ; L₆: titer 10^{-4} ; L₇: titer 10^{-5} ; L₈: titer 10^{-6} .

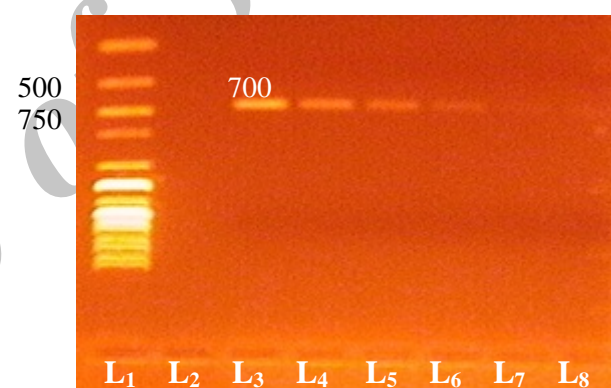


Figure 3. Sensitivity of PCR in spore
L₁: molecular size marker (1-kbp ladder); L₂: negative control; L₃: titer 10^{-1} ; L₄: titer 10^{-2} ; L₅: titer 10^{-3} ; L₆: titer 10^{-4} ; L₇: titer 10^{-5} ; L₈: titer 10^{-6} .

DISCUSSION

Honeybees are attacked by numerous pathogens including viruses, bacteria, fungi and parasites. One of the most economically important diseases of honey bees is the bacterial diseases American foulbrood (AFB). AFB is causing considerable economic loss to beekeepers all over the world. The gold standard for bacterial identification is usually the isolation of bacterium. However, this may not be feasible for a pathogen like *Paenibacillus larvae*. Isolation and subsequently biochemical identification can take up to two weeks.

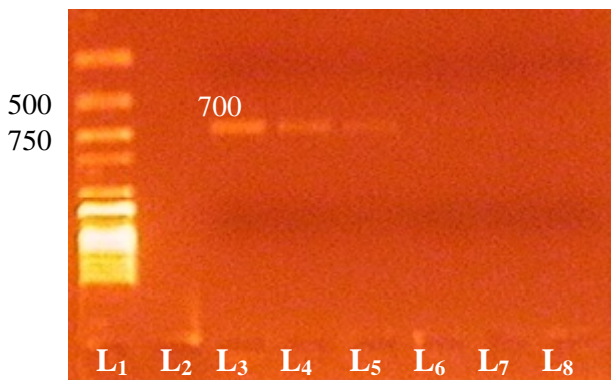


Figure 4. Sensitivity of PCR in experimentally contaminated honey

L₁: molecular size marker (1-kbp ladder); L₂: negative control; L₃: titer 10^{-1} ; L₄: titer 10^{-2} ; L₅: titer 10^{-3} ; L₆: titer 10^{-4} ; L₇: titer 10^{-5} ; L₈: titer 10^{-6} .

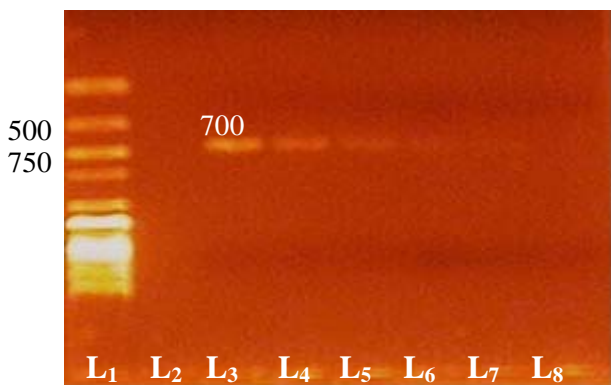


Figure 5. Sensitivity of PCR in experimentally contaminated larvae

L₁: molecular size marker (1-kbp ladder); L₂: negative control; L₃: titer 10^{-1} ; L₄: titer 10^{-2} ; L₅: titer 10^{-3} ; L₆: titer 10^{-4} ; L₇: titer 10^{-5} ; L₈: titer 10^{-6} .

Nowadays, molecular markers, such as the 16S rRNA gene, have been extensively applied to investigation of the occurrence and distribution of bacteria in environmental samples. PCR is a reliable, fast and widely used method in microbiological diagnosis and the testing of DNA from pathogens is an alternative to the classic cultivation tests on agar. The aim of this study was to create a fast and sensitive method based on PCR for the detection of *Paenibacillus larvae larvae* from a bacterial colony, larvae and honey that does not require a long time to process culture.

Govan et al. 1999 described a PCR detection technique that could be used on *Paenibacillus larvae* colonies that are able to grow on semi-selective media. The sensitivity of this method was 50 cfu /ml. Piccini et al. 2002, reported the use of polymerase chain reaction (PCR) to detect *Paenibacillus larvae larvae* spores from in vitro cultures, larvae with clinical symptoms and also artificially contaminated honey. The sensitivity of this method was 32 cfu /ml for spore and 170 cfu /ml for artificially contaminated honey. Lauro et al. 2003, described a touchdown nested PCR to detect the presence of *Paenibacillus larvae pulvifaciens* spores directly in honey and hive samples. The sensitivity of this method was 10 cfu /ml, but this technique does not allow discrimination between *Paenibacillus larvae larvae* and *Paenibacillus larvae pulvifaciens*. Alipi et al. 2002 provided a method using PCR and RFLP that could differentiate between *Paenibacillus larvae larvae* and *Paenibacillus larvae pulvifaciens*. Alipi et al. 2004 could differentiate *Paenibacillus larvae larvae* and *Paenibacillus larvae pulvifaciens* by using ERIC-PCR. This technique could be directly used for honey and brood samples. The sensitivity of this method was 283 cfu /ml.

In this study, PCR was performed, using bacterial cells, spore form, and experimentally contaminated honey and larvae with spore, using primers based on the 16S rRNA gene. Primers used were highly specific for *Paenibacillus larvae larvae*, and did not have cross-reactions with a large number of bacterial species commonly found in hive samples. Since PCR sensitivity is very important for diagnostic purposes, detection limit was determined at every step. For this purpose, we prepared serial dilutions from 10^{-1} to 10^{-6} of bacterial and spore suspension then performed PCR and plate count for each dilution. The low number of bacteria and spores that we were able to detect in pure form of them were 28 cfu /ml for bacterium and 33 cfu /ml for spore. The low number of spores that we were able to detect in experimentally contaminated honey was 330 cfu /ml and in 243 cfu for larval remains. In the sensitivity test for bacterium, Govan could detect 50

cfu /ml whereas this study detected 28 cfu /ml. This difference may be related to the design and sensitivity of primers. In the sensitivity test for spore, Piccini detected 31 cfu /ml which is almost similar to the number of 33 cfu /ml detected in this study. In the sensitivity test for contaminated honey, Piccini detected 170 cfu /ml, whereas 313 cfu /ml were detected in this study. Although primers used here, were similar to Piccini, the DNA extraction methods were different. In addition, the sensitivity test results for contaminated honey were different from the result of other studies because, the methods and primers had different sensitivities. As a conclusion, this method was set up for the first time in Molecular Biology Laboratory of Honey bee Department in Razi Vaccine and Serum Research Institute. This PCR technique can be used for detection of the presence of *Paenibacillus larvae larvae* spores in honey samples, sub-clinical brood samples and after cultured medium without the need for biochemical tests. Also, it may be used for the examination of honey samples for export and import in order to detect *Paenibacillus larvae larvae* spores, which is often necessary. Due to the increased number of AFB-affected apiaries of Iran, and considerable amount of the cost and time of a microbiological diagnosis, a rapid and accurate method for the detection of *Paenibacillus larvae larvae* is quite necessary.

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