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Full Article

Detection of *Paenibacillus larvae larvae* spores in honey and diseased larvae Samples by culture and PCR

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ABSTRACT

American foulbrood (AFB) is the most serious brood disease of the honey bee. Traditional methods are reliable but rather slow simply because they are based on biochemical, morphological and physiological identification of cultivated isolates. The aim of this study is the detection of *Paenibacillus larvae larvae* spores in honey and diseased larvae samples by culture and PCR. Therefore 54 samples of diseased larvae and 36 honey samples, were diluted with an equal volume of distilled water and centrifuged, then the pellet was used for bacterial culture, DNA extraction and PCR. PCR products were electrophoresed on 0.8 % agarose gel. Five of 54 (9.3 %) larvae samples and 5 of 36 (13.9 %) honey samples were positive for *Paenibacillus larvae larvae* by culture and PCR. As a result, screening of honey and larvae samples by PCR method proved to be a reliable, fast and useful method on regional and national scale for monitoring, controlling, and using preventive measures before the occurrence of American foulbrood damages.

Keywords: Hony bee, Paenibacillus Larvae, American Foulbrood(AFB), Brood disease, PCR

INTRODUCTION*

American foulbrood (AFB) is the most serious brood disease of the honey bee. The disease is caused by the Gram-positive spore-forming bacterium Paenibacillus larvae larvae (Ashiralieva & Genersch, 2006, Genersch *et al* 2006). AFB is spread vertically in colonies and horizontally by means of bee trade, contaminated equipment or insertion of used or contaminated brood or honeycombs into the hives (Fries *et al* 2006). This cosmopolitan and highly infectious disease affects larval and pupal stages during the honeybee development. Dead larvae decompose to a glue-like colloid liquid, producing a specific smell. The bacterium creates spores covered by seven protective

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layers which enable it a life-span of at least 30–50 years (Bakhiet & Stahly 1985).

Nursebees try to remove this scale from cells and spread spores to other larvae. The disease is so infectious that it can destroy the infected honeybee colony within a few months or years (Hansen & Brodsgaard1999). Traditional methods are reliable but rather slow simply because they are based on biochemical and morphological/physiological identification of cultivated isolates (Reynaldi & Alippi, 2006). This method also requires relatively long cultivation. The aim of this study was to assess the feasibility of PCR-amplified 16S rDNA for the identification of P. larvae subsp. larvae from other Paenibacillus organisms and from other spore-forming bacteria from apiarian sources and to assess its applicability to the direct and rapid diagnosis of AFB.

MATERIALS AND METHODS

Sampling. The 54 samples of diseased larvae and 36 honey samples used in this study were obtained from Honeybee, Silkworm and Wildlife Research Department, from 1388 till 1389 and from different parts of the country.

Sample preparation:

Diseased larvae. Remains of 4 diseased larvae were suspended in 2 ml of sterile distilled water (SDW) and mixed thoroughly and centrifuged for 5 min at 484 g. The supernatant was centrifuged for 30 min at 6000 g, and then pellet containing the spores was suspended in 2 ml of SDW. One ml was used for culture and 1 ml for DNA extraction and PCR (Antunez *et al* 2004, Alessandro *et al* 2007).

Honey. Forty grams of honey were mixed with 40 ml of sterile distilled water and centrifuged at 6,000 g for 45 min, and then pellet containing the spores was suspended in 2 ml of SDW. One ml was used for culture and 1 ml for DNA extraction and PCR (Antunez *et al* 2004, Alessandro *et al* 2007).

Culture. Spore suspension was heated at 80 °C for 15 min. Then 1 ml was inoculated to 5 plates (200 µl per each plate) of MYPGP agar (Mueller-Hinton broth 10 g, yeast extract 15 g, K_2PO_4 3 g, glucose 2 g, Na-pyruvate 1 g, agar 20 g and SDW 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 % CO_2 [(Antunez et al., 2004) & (Alessandro B.D et al., 2007) & (OIE Manual, 2010)].

Identification. On MYPGP agar, colonies are small, regular, mostly rough, flat or raised and whitish to beige coloured. Gram staining, Catalase, Biochemical tests (Production of acid from carbohydrates, Hydrolysis of casein, Hydrolysis of starch, Liquefaction of gelatin, Indole, Motility) were used for identification (OIE Manual, 2010).

Bacterial strains. *Paenibacillus larvae larvae* standard strain was obtained from Pasteur Institute of France.

[CIP 104618: ATCC 9545 = CCUG 28515 = DSM 7030 = LMG 9820].

DNA extraction of bacterium. A colony of *Paenibacillus larvae larvae* isolate was suspended in 50 µl of SDW and heated at 95 °C for 15 min. After centrifugation at 5000 g for 5 min, the supernatant was used as DNA template for PCR [(Govan et al., 1999) & (Neuendorf et al., 2004)].

DNA extraction of spore. 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 water bath, mixing every 10 min. (Vary 1973, Paidhungat & Setlow 2000). After incubations, the samples were washed with P.B.S. 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. 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 12000 g. 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 (Bickley & Owen 1995, Alessandro *et al* 2007).

PCR conditions. The specific primers used, were described by Piccini et al. (2002). Primers were Pl_5: 5′-CGAGCGGACCTTGTGTTTCC-3, Pl_4: 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 $MgCl_2$. All PCR materials were from Fermentas company.Thermal cycling included the following steps: 95 °C (1 min); 30 cycles of 93 °C (1 min), 55 °C (30 sec), 72 °C (1 min); and a final cycle of 72 °C (5 min), using an eppendoref mastercycler gradiant thermocycler. PCR products were visualized by electrophoresis in 0.8% (w/v) agarose gel stained with ethidium bromide. Product size was determined using the 1-kb molecularsize ladder (Piccini *et al* 2002, Sambrook *et al* 1989).

Sensitivity and specificity of the PCR assay. Sensitivity and specificity tests were done according to Modirrousta & Moharrami (1389). To investigate the practical value of the sensitivity in honey and larvae samples, serial spore dilutions of 10^{-1} to 10^{-6} were prepared and 1ml of each dilution was added to 20 gr honey and 2 healthy larvae, and DNA extraction and PCR were performed. One ml of each dilution was used for culture plate count.

To reveal the specificity of the PCR, reactions were tested with the following bacterial strains: Paenibac*illus larvae standard, Paenibacillus larvae larvae wild, Bacillus cereus, Bacillus subtilis, Listeria monocytogenes, Staphylococcus epidermidis, Escherichia coli, Salmonella entritidis, Citrobacter freundii, Entrobacter sakazakii, Klebsiella pneumonia* and *Morganella morganii.*

RESULTS

The result of PCR specificity, determined that 2 out of 12 bacterial strains were positive which were related to *Paenibacillus larvae larvae* standard strain and wild strain. The result of PCR sensitivity, in artificially contaminated honey and larvae were 330 and 243 cfu /ml at 10^{-3} dilutions.

From 36 honey samples tested for AFB, five were positive (13.9 %) using cultivation tests. Positivity was confirmed by PCR tests (Table 1). From 54 samples of diseased larvae tested for AFB, five were positive (9.3 %) using cultivation tests. Positivity was confirmed by PCR tests (Table 2)**.**

The results of PCR for *P.l.l* on colonies growth on agar, showed that 5 honey samples were positive (Figure 1).

The results of PCR for the presence of P.l.l spores in honey and larvae direct samples, showed that 5 larvae and 5 honey samples were positive (Figure 2).

The results of biochemical tests for 10 positive isolates of *P.l.l*, all 10 isolates were similar to the standard strain (Table 3).

DISCUSSION

The American foulbrood (AFB) is an infectious and highly contagious disease of honey bee brood (Apis mellifera) and other Apis species. Bee colonies with good hygienic behavior are able to quickly eliminate infected larvae and consequently eradicate large parts of a potential infection source. The progress of the disease in colonies with good hygienic behavior is much slower. Recognition of the AFB in apiaries should be confirmed with laboratory tests. The early detection of American foulbrood is possible by examination of stored food and extracted honey. Honey samples are especially suitable for a screening of AFB in a larger area.

Table 1. Results of culture and PCR on honey samples.

Sample	Culture/P.l.l	Cfu / ml	PCR	Sample	Culture/P.l.l	Cfu / ml	PCR
H1	NG	0	-	H19	NG	0	-
H2	-	3	-	H20	-	33	-
H3	-	8	-	H21	NG	0	-
H4	-	20	-	H22	NG	0	-
H5	NG	0	-	H23	-	1	-
H6	NG	0	-	H24	+	447	+
H7	-	6	-	H25	NG	0	-
H8	NG	0	-	H26	NG	0	-
H9	-	3	-	H27	NG	0	-
H10	NG	0	-	H28	NG	0	-
H11	NG	0	-	H29	NG	0	-
H12	-	4	-	H30	NG	0	-
H13	NG	0	-	H31	NG	0	-
H14	NG	0	-	H32	+	510	+
H15	NG	0	-	H33	+	380	+
H16	NG	0	-	H34	NG	0	-
H17	NG	0	-	H35	+	669	+
H18	NG	0	-	H36	+	465	+

Table 2. Results of culture and PCR on Larvae samples

Sample	Culture/P.l.l	Cfu /ml	PCR	Sample	Culture/P.l.l	Cfu /ml	PCR
L1	NG	0	-	L28	NG	0	-
L2	NG	0	-	L29	-	1	-
L3	NG	0	-	L30	NG	0	-
L4	NG	0	-	L31	-	6	-
L5	NG	0	-	L32	NG	0	-
L6	NG	0	-	L33	NG	0	-
L7	NG	0	-	L34	NG	0	-
L8	NG	0	-	L35	NG	0	-
L9	NG	0	-	L36	NG	0	-
L10	NG	0	-	L37	NG	0	-
L11	-	7	-	L38	NG	0	-
L12	NG	0	-	L39	NG	0	-
L13	NG	0	-	L40	NG	0	-
L14	NG	0	-	L41	NG	0	-
L15	NG	0	-	L42	NG	0	-
L16	NG	0	-	L43	NG	0	-
L17	NG	0	-	L44	NG	0	-
L18	NG	0	-	L45	+	661	+
L19	NG	0	-	L46	-	21	-
L20	NG	0	-	L47	-	5	-
L21	NG	0	-	L48	+	506	+
L22	NG	0	-	L49	NG	0	-
L23	-	16	-	L50	NG	0	-
L24	-	28	-	L51	NG	0	-
L25	NG	0	-	L52	+	437	+
L26	NG	0	-	L53	+	755	+
L27	NG	0	-	L54	+	288	+

(NG: No Growth, - : No Growth *P.l.l*)

Table 3. Results of biochemical tests.

Sample	Catalase	SIM	D (+) glucose	D (+) trehalose	D (+) xylose	L (+) arabinose	Hydrolysis of casein	Hydrolysis of starch	Liquefaction of gelatin
Standard strain	-	---	+	+	-	-	+	-	+
L_{-45}	-	---	+	+	-	-	+	-	+
L_{-48}	-	---	+	+	-	-	+	-	+
L_{-52}	-	---	+	+	-	-	+	-	+
L_{-53}	-	---	+	+	-	-	+	-	+
L_{-54}	-	---	+	+	-	-	+	-	+
H_{24}	-	---	+	+	-	-	+	-	+
H_{32}	-	---	+	+	-	-	+	-	+
H_{33}	-	---	+	+	-	-	+	-	+
H_{35}	-	---	+	+	-	-	+	-	+
H_{36}	-	---	+	+	-	-	+	-	+

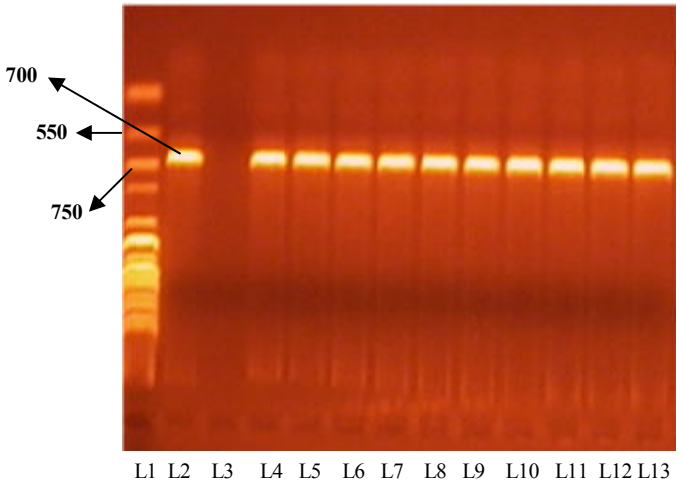

Figure1. PCR result in colony growth on media.
L1= Ladder 1 Kb ; L2= *Paenibacillus larvae standard* ; L3= C^- ; L4= L_{45} ; L5= L_{48} ; L6= L_{52} ; L7= L_{53} ; L8= L_{54} ; L9= H_{24} ; L10= H_{32} ; L11= H_{33} ; L12= H_{35} ; H_{36}

An early diagnosis of AFB even in its sub-clinical stage, is therefore of great advantage. Another essential advantage consists in the surveillance of sanitation measures.

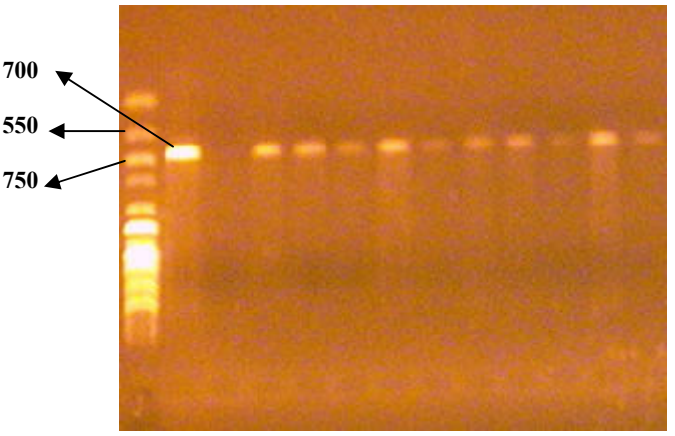

Figure 2. Direct PCR result in honey and larvae samples.
L1= Ladder 1 Kb ; L2= *Paenibacillus larvae standard* ; L3= C^- ; L4= L_{45} ; L5= L_{48} ; L6= L_{52} ; L7= L_{53} ; L8= L_{54} ; L9= H_{24} ; L10= H_{32} ; L11= H_{33} ; L12= H_{35} ; L13= H_{36}

Inadequate sanitation of an apiary as well as undetected centers of epidemics can be recognized from the increasing spore contents. According to Ritter's research (2003) only 2% of honey samples from German apiaries contained *Paenibacillus larvae* spores. The spores were detected in 98 % of the 700 samples of honey imported to Germany from non-EU countries and in 62 % (from 200 tested) of honey samples from the EU. Similar research was done in Germany in 1993-1996 by Von der Ohe (1997). Following tests of 2099 samples of German and foreign honey, research results showed the presence of P. larvae in only 7 % of German honey samples while all the foreign samples (from Argentina, Greece, Iran, Russia and US) had very high contents of P. larvae spores. Lipinski et al. (2007) reported that 51 honey samples out of 251 (20.3 %) were positive. Antunez et al. (2004) in 2001- 2002 tested 101 honey samples, from 19 provinces *P.larvae* was found in 52 samples (51.5%). Graaf et al. (2001) showed that 11% of the 1328 Belgium honey samples tested were infected with *Paenibacillus larvae larvae*. In this study, 36 honey samples and 54 suspected diseased larvae samples which were obtained from different apiaries for analytical and laboratory diagnostic purposes, were cultivated for American foulbrood. From 5

honey samples (13.9%) and 5 suspected diseased larvae (9.3%) samples, *Paenibacillus larvae larvae* was isolated. All 10 samples were positive by PCR too. The number of spores that were detected by plate count was 380- 669 cfu/ml for honey and 288-755 cfu/ml for larvae. The sensitivity of PCR for honey and larvae were 330, 243 cfu/ml, whereas Alippi et al determined sensitivity of PCR 283 cfu/ml and Piccini et al. determined 170 cfu/ml. Differences among sensitivity results, could be a result of different methods of DNA extraction and primer sensitivity. The results of this study showed that, when the plate counts were higher than the sensitivity test, the PCR was positive. All positive samples in culture were positive in PCR too.

PCR can be used for the detection of P. larvae spores in honey samples or sub-clinical brood samples or after culturing on a growth medium without the need for biochemical tests. The method used in the research allows detection of sub-clinical infections. In many apiaries, the outbreak of AFB (clinical symptom) can be avoided by early preventive action.
Tests of honey (food source) for presence of the *Paenibacillus larvae* can detect the infection in a bee colony before the outbreak of the disease and its clinical symptoms. The detection method allows for early preventative treatment against the development and spread of the disease. The detection method helps reduce the cost and workload of fighting AFB.

The results show that cases of the AFB disease are actually higher than the number of official notifications to the veterinary inspector may suggest.
The test results point at a high advisability of systematic monitoring of the AFB in Iran. Such an undertaking is necessary to assess the epizootic situation of the AFB in apiaries. Systematic monitoring will allow for improvement of existing conditions. It can lower the risk of the disease development, without to destroying the bee colonies.
As a result, there may be a correlation between the level of *Paenibacillus larvae* infection of honey and occurrence of AFB symptoms in honey bee colonies.

The diagnosis of AFB from clinical symptoms implies the disadvantageous since it can only be done after the outbreak of the disease and therefore excludes the possibility to recognize sub-clinical stages, facilitating prophylaxis or controlling sanitary measures.

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