

MOLECULAR DIAGNOSIS OF AMERICAN FOULBROOD IN HONEYBEE BROOD IN BULGARIA

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Abstract

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The analysis of the 47 isolates for identification of *Paenibacillus larvae*, obtained of honeybee brood with American foulbrood (AFB) symptoms from 9 different districts of Bulgaria by conventional PCR technique was performed. Specific DNA products by size 1106 bp were obtained from the 42 isolates. Five isolates were PCR negative for AFB (*P. larvae*). The results showed that PCR assay is specific and sensitive method for molecular identification of *Paenibacillus larvae* and can provide a rapid and reliable diagnosis of infected honeybee brood.

Key words: Honeybee brood, AFB, *Paenibacillus larvae*, PCR

Introduction

American foulbrood (AFB) is a disease of bee brood caused by the spore-forming bacterium *Paenibacillus larvae*. This is the most serious disease of bee brood, leading to significant economic losses to beekeepers worldwide. The main host of the bacterium is the honeybee *Apis mellifera* (Lindström and Fries, 2005). Spores of *P. larvae* can be isolated from honey, wax, pollen, walls of an infected beehive and debris (Gochbauer and Corner, 1987). These spores can remain infectious for long - at least 35 years (Haseman, 1991), but there have been reports of a longer period of survival (Borchert, 1979).

The disease can be spread by spores when transporting bees, queen bees and bee hives, as well as theft, or using tools and inventory, beekeeper clothes and contaminated pollen or honey (Gochbauer and Corner, 1987). There are different culture media, developed for isolation of *P. larvae*, but other similar microorganisms grow on them as well, which complicated the timely diagnosis.

Spores of *Paenibacillus larvae* and slow-growing vegetative forms of this bacterium are difficult for primary isolation and its identification takes a long time (Dobbelaere et al., 2001). The finding of spores in naturally infected bee larvae is very important to confirm the presence of the disease

(Piccini et al., 2002). Therefore, a new strategy is needed in the fight against American foulbrood and early diagnosis on subclinical level.

In recent years, other methods such as ELISA and polymerase chain reaction (PCR) are successfully applied in the diagnosis of many bacterial, viral and parasitological diseases, which enable the accurate and quick detection and differentiation of pathogens (Avaniss-Aghjani et al., 1994; Dobbelaere et al., 2001; Piccini et al., 2002).

The purpose of this paper is the proving of *P. larvae*, isolated from diseased brood by conventional PCR technique in order to introduce the method into routine laboratory diagnosis of American foulbrood in Bulgaria.

Materials and Methods

Forty-seven isolates of honeycomb with brood, suspicious for American foulbrood (AFB), were investigated. Samples were sent by 9 different regions in Bulgaria: Burgas, Veliko Tarnovo, Vratsa, Gabrovo, Pernik, Plevan, Smolyan, Sofia and Yambol.

Samples were previously studied bacteriologically on blood agar and selective MPYGP agar. For the determination of the obtained isolates as *Paenibacillus larvae*, they were also tested with AFB kit "Vita. Europe", based on monoclo-

nal antibodies. These isolates were then examined with conventional PCR technique using protocols recommended in the OIE Terrestrial Manual (OIE, Terrestrial Manual, 2008). The target DNA needed for PCR was prepared from the bacterial culture by dissolving one colony in 50 µl of distilled water, heating at 95°C/15 min and centrifugation at 5000 g/5 min. The supernatant containing DNA was used in PCR (Govan et al., 1999). The study was conducted with a pair of specific primers (AFB-F and AFB-R) with the following nucleotide sequence:

AFB-F-(5'-CTT-GTG-TTT-CTT-TCG-GGA-GAC-GCC-A-3') and

AFB-R-(5'-TCT-TAG-AGT-GCC-CAC-CTC-TGC-G-3').

The reaction was conducted in a volume 25 µl per sample. The reaction mixture contained: primers AFB F and AFB R (x 50pM); dNTPs (x 200 µl of each dNTP); PCR buffer (x 2.50 µl of the kit); Tag Polymerase (x 0.5 U); a target DNA (x 5.0 µl of goods) and distilled water (dH₂O) to 25 µl (Govan et al., 1999).

Proving specific DNA was performed by amplification of genomic DNA of *Paenibacillus larvae* in a thermocycler "Techne TC-412" (UK) with the following temperature regime: 1 cycle *denaturation* at 95°C for 15 min and 30 cycles consists of: *melting* at 93°C for 1 min; *annealing* at 55°C for 30 s and *extension* of 72°C for 1 min. Final extension cycle (*elongation*) at 72°C for 5 min. The PCR products were visualized by horizontal electrophoresis on a 2% agarose gel at 100 V for 30 min, stained in ethidium bromide solution (1:10000) for 10 min and photo-documented on UV-transilluminator with camera VisiDoc-It (UVP, USA).

Results and Discussion

Most of the tested samples were with typical clinical signs of AFB – mottled brood, the capping of cell are punctured, becomes darkened and concave, the larvae become glutinous in consistency (Figure 1).

Bacteriological inoculations on blood agar and selective MPYGP agar showed growth of *Paenibacillus larvae* in 42 of the samples (Figure 2). When performed with AFB kit ELISA assay, positive results were seen in the same 42 isolates of *Paenibacillus larvae* (Figure 3).

We received specific DNA product with a size of 1106 bp from 42 examined by PCR isolates (Figure 4, lines 1-15; Figure 5, lines 1-6). These results confirmed the previously conducted bacteriological examination and testing with AFB kit (Table 1). The varying intensity of the observed bands may be due to the different numbers of the nucleotide sequences located along the length of the DNA chain of *Paenibacillus*

larvae. No PCR amplicons were received from five isolates from the regions Burgas (2), Gabrovo (2) and Smolyan (1) (Figure 5, lines 7-11). The results of the application of our PCR method for identification of *P. larvae* were confirmed when carried out by the EURL in France interlaboratory tests for bee health in April 2014. These and our previous stud-



Fig. 1. Clinical picture of AFB diseased bee brood

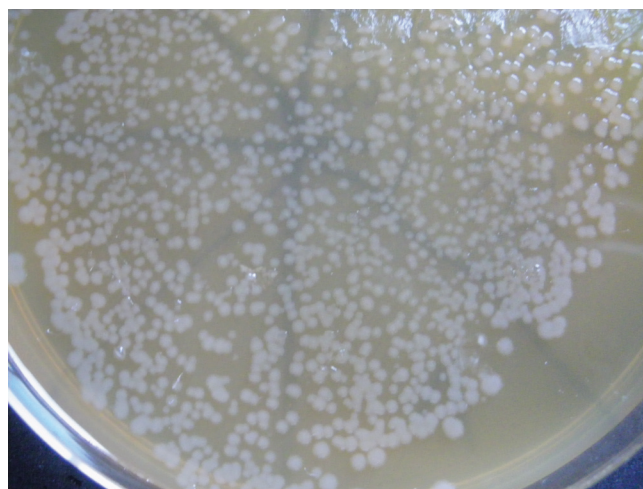


Fig. 2. Culture of *Paenibacillus larvae* on the MPYGP agar

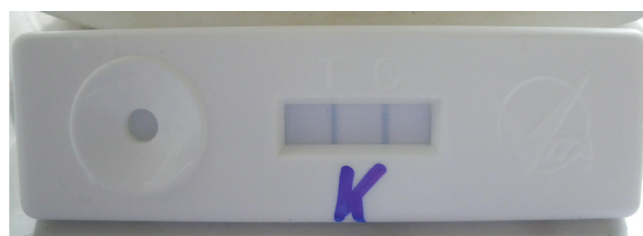


Fig. 3. Positive AFB test

Table 1
Isolates from diseased brood tested for *Paenibacillus larvae*

Area from which the samples are taken	Number of analyzed samples	Number of samples positive for <i>Paenibacillus larvae</i>		
		Microbiological	AFB kit	PCR
Burgas	15	13	13	13
Veliko Tarnovo	1	1	1	1
Vratsa	1	1	1	1
Gabrovo	13	11	11	11
Pernik	2	2	2	2
Pleven	6	6	6	6
Smolyan	5	4	4	4
Sofia region	2	2	2	2
Yambol	2	2	2	2
Total	47	42	42	42

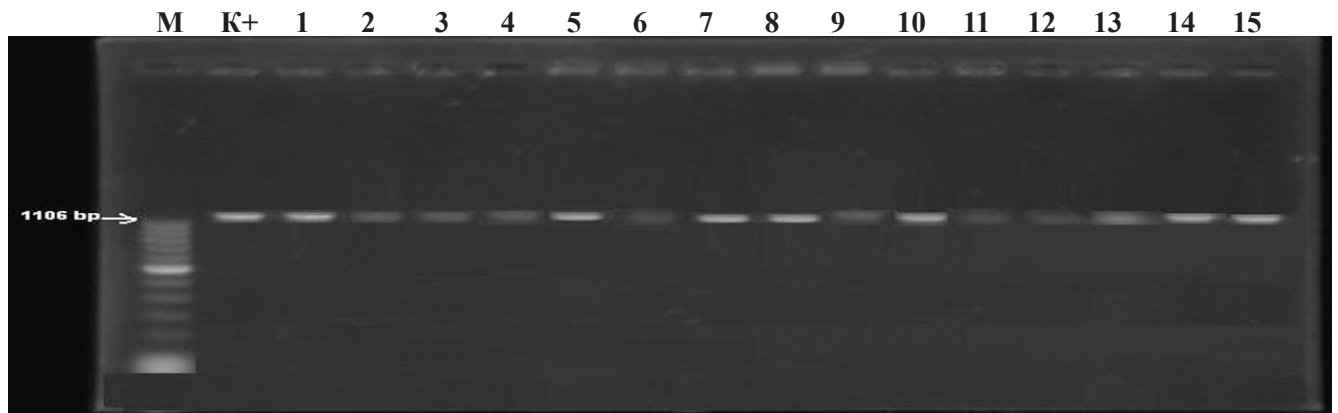


Fig. 4. Results from PCR testing of the strains, isolated from AFB diseased brood

Legend: M - marker 100 bp; K+ - control, lines 1-15 - PCR amplicons with different intensity and size of the bands 1106 bp

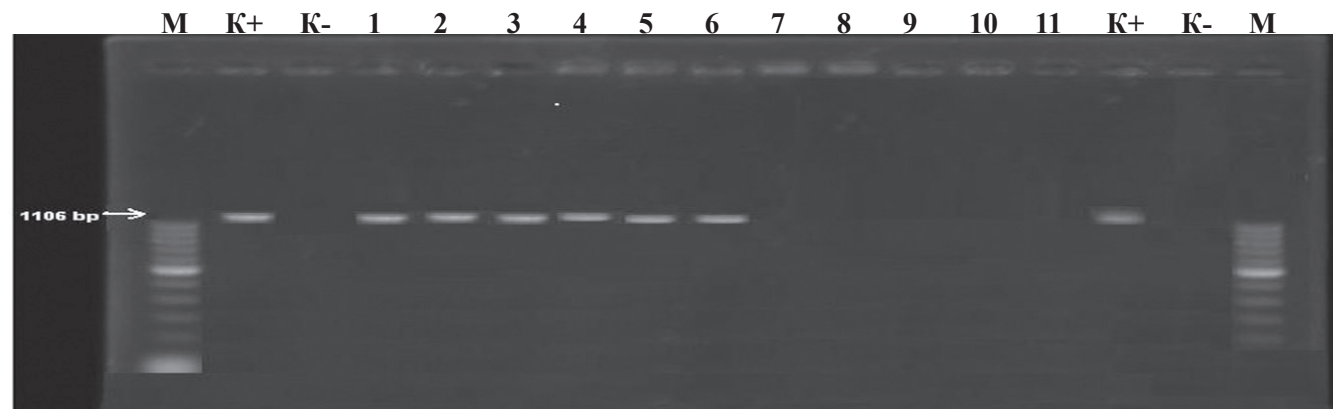


Fig. 5. Results from PCR testing of the strains, isolated from AFB diseased brood

Legend: K(+) - control, K(-) – control (H₂O); lines 1-6 - PCR amplicons with size of 1106 bp; lines 7-11 - absence of PCR amplicons, M - marker 100 bp;

ies (Gurgulova et al., 2013) indicate that the method is sufficiently specific and is suitable for confirmation of positive bacteriological isolates and rapid identification of *P. larvae* in samples of brood, honey and bees. Other authors reported similar results. Using PCR method Piccini et al. (2002) detected spores of *P. larvae* in naturally infected bee larvae and artificially contaminated honey. Govan et al. (1999) tested PCR primers designed on the basis of 16S rRNA gene of *P. larvae*, on five types of bacteria: *P. alvei*, *P. polymyxa*, *B. pumilus* and *B. subtilis*, isolated from honeybees. Specific PCR product size 973 bp is able to obtain from *P. larvae*, but not by other bacterial species.

Dobbelaere et al. (2001) investigated with PCR 14 bacterial species associated with the beehive, using two pairs of primers in 4 different combinations and all of them received amplicates with adequate size only from *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens*, and from DNA extracted directly from the remains of AFB diseased larvae.

Ash et al. (1993) after molecular rRNA identification of three *Bacillus* spp. by PCR test, proceed with a proposal to create a new genus *Paenibacillus*. De Graaf et al. (2006), Qin et al. (2006) and Genersch et al. (2006) used PCR methods for the identification of *P. larvae*, as well as for determining the genotype of the pathogen which makes it possible the subspecies *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens* be reclassified into a single species - *Paenibacillus larvae*.

The obtained results of our study, similar to those of the quoted authors, clearly demonstrate that PCR analysis was specific and sensitive method for identification of *Paenibacillus larvae*, and can provide rapid and reliable diagnosis of AFB infected brood within only a few hours. This method can be appropriate for the early diagnosis of AFB in a study of adult bee (Gurgulova et al., 2013), honey bees and the debris from the bottom of the beehive (Gochnauer and Corner, 1987).

Conclusions

PCR analysis coincides and confirms the positive results from bacteriological and AFB test for the identification of *Paenibacillus larvae*.

The PCR test is sufficiently fast, specific and sensitive method for the identification of *P. larvae*. It is suitable for introduction into laboratory practice in our country for early diagnosis of AFB in honeybee brood.

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