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Original article

DETECTION OF *PAENIBACILLUS LARVAE* SPORES IN HONEY BY CONVENTIONAL PCR AND ITS POTENTIAL FOR AMERICAN FOULBROOD CONTROL

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Summary

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The present study attempted to detect *Paenibacillus larvae* spores in naturally contaminated honeys by conventional PCR and to determine the sensitivity of the reaction with different primer pairs in order to assess its potential for American foulbrood control. For this purpose, duplicated honey samples were collected from 5 bee colonies with clinical American foulbrood and 5 clinically healthy colonies in the same apiary. The samples were analysed for the presence of *Paenibacillus larvae* spores by culture method and subsequent PCR detection in bacterial colonies. The PCR performed directly with spore DNA failed in 6 out of the 20 honeys investigated with spore load of 10–46 cfu/g. The established sensitivity of 70% of the reaction in the present study shows that the adequate control of American foulbrood by analysis of honeys for *Paenibacillus larvae* spore contamination should be done by combination of culture method followed by PCR in bacterial colonies, whose sensitivity was 100%.

Key words: American foulbrood, control, honey, *Paenibacillus larvae*, PCR, spores

INTRODUCTION

Paenibacillus larvae (*P. larvae*) is the causative agent of American foulbrood on larvae of honey bees *Apis mellifera* and *Apis cerana* (Genersch, 2008). This is the most severe disease of bacterial origin leading to significant losses of beekeeping worldwide (Genersch, 2010).

An important characteristic of the agent is the ability to produce spores which seem to be the only infectious form (Bakonyi *et al.,* 2003). Spores withstand the adverse environmental conditions for 35–50 years (Bakhiet & Stahly, 1985). Spores of the pathogen can be found in honey, wax, pollen, bees and on the hive walls and remains of wintering bees (Piccini *et al*., 2002; Ryba *et al.*, 2009). This determines the extreme contagiosity of

American foulbrood and its rapid spread in the apiary in the routine apicultural practice. The untimely diagnosis of disease and non-implementation of measures for control inevitably brings about death of affected bee colonies, as well as of entire apiaries (Matheson & Reid, 1992).

American foulbrood control is conducted with different methods in the countries. In the European Union the administration of antibiotics and sulfonamides in beekeeping is prohibited, while in USA, Canada, Argentina their use is allowed (Bogdanov, 2006). The main methods for control of the disease include: destruction of the affected bee colonies (radical method); shaking method (or artificial swarm) (Hansen & Brodsgaard, 2003; Parvanov *et al.,* 2006) and medical treatment. When these methods are applied independently without laboratory examination of clinically healthy bee colonies for honey contamination with *P. larvae* spores, they are not reliable.

P. larvae sporulates readily in infected bee larvae but hardly in laboratory conditions. In the last decades, different culture media supporting the growth and germination of *P. larvae* spores, have been developed (De Graaf *et al.,* 2006). Traditional identification methods are not fast enough and are based on the culture of materials with subsequent morphological, biochemical and physiological identification of the isolates (Rusenova *et al.,* 2013).

Scientific papers about detection of the causative agent of American foulbrood by polymerase chain reaction are still few. Piccini *et al.* (2002) proved *P. larvae* in honey artificially contaminated with spores. Lauro *et al.* (2003) developed a protocol for establishment of *P. larvae* spores in honey by a novel nested PCR. The proposed by the authors nested PCR permits detection of the spores at subclinical level but this technique requires a special laboratory equipment and shows tendency to easier contamination of the reaction mixtures. Bakonyi *et al.* (2003) and D'Alessandro *et al.* (2007) compared different methods for DNA extraction from spores of *P. larvae* in honey and adult bees.

The present study attempted to detect the spores of *Paenibacillus larvae* in naturally contaminated honeys by conventional polymerase chain reaction and to determine the sensitivity of the reaction with different primer pairs in order to assess its potential in American foulbrood control.

MATERIALS AND METHODS

Analysis of honey samples for P. larvae spore contamination by culture method and PCR

Honey samples originated from bee colonies with clinical American foulbrood (n=5) and from clinically healthy colonies (n=5) from the same apiary. Honey samples from each colony were obtained in duplicate. Materials were processed according to the protocol described by Iurlina & Fritz (2005) with some modifications. Each sample of 10 g was diluted 1:2 (w/v) in phosphate-buffered saline (PBS, pH 7.2) and centrifuged at 4000 ×*g* for 30 min. The supernatant was discarded leaving 1 mL to suspend the gel. Then, the suspension was heat treated at 85 ºC for 15 min and 100 µL were inoculated on Petri dishes with suitable agar medium and cultured at 37 ºC under aerobic conditions for up to 6 days. Suspect for *P. larvae* colonies were identified with multiplex PCR for detection of the agent in isolated bacterial colonies (Rusenova *et al.,* 2013). The amount of viable spores per gram honey was determined by the agar spread method (Markey *et al.,* 2013).

P. larvae spore DNA extraction method

Ten grams of honey were mixed with 10 mL of sterile distilled water (SDW), centrifuged at 6000×*g* for 45 min and then the depot of spores was suspended in SDW. Spore suspensions were centrifuged at 6000×*g* for 30 min to concentrate the spores. Depot was incubated in buffer containing 0.1 M NaOH; 0.1 M NaCl (pH 10.8); 1% SDS (w/v, Sigma) and 0.1 M DTT (Sigma) for 30 min at 70 °C water bath with shaking every 10 min. Further the samples were treated with lysozyme (Sigma, 1.5 $g.L^{-1}$ final concentration in TE buffer) for 1 h at 37 °C, with SDS (1 % w/v) and proteinase K 0.2 $g.L^{-1}$ (Fermentas). Proteins were precipitated with ammonium acetate (2.5 M) and 100 % cold ethanol was used for nucleic acids precipitation (D'Alessandro *et al.,* 2007). All samples were re-suspended in 100 µL TE and used as DNA template for PCR.

Detection of P. larvae spore DNA by PCR

Different primer sequences, available in the specialised literature, were tested for amplification of 16S rRNA and MlP gene segments of bacterial and spore *P. larvae* DNA (Table 1). PCR was run at temperature conditions and reagents' concentrations as previously described (Rusenova *et al.,* 2013). A reference strain *P. larvae* NBIMCC 8478 was used as a positive control in each experiment.

The presence of amplification products was visualised by electrophoresis in 1.5% and 2% agarose gels, supplied by StarLab (Sigma-Aldrich).

The sensitivity of the reaction for detection of spore DNA was determined by ten-fold dilutions of DNA (10^{-1} to 10^{-3}). PCR experiments for detection of spore DNA and sensitivity of the reaction were carried out twice to prove the method's reproducibility.

RESULTS

All of the 20 honey samples analysed with the culture method with subsequent PCR detection in isolated bacterial colonies, showed positive result for contamination with *P. larvae* spores at a level from 10 to 10^5 cfu/g honey.

The attempts for DNA isolation from spores led to amplification of the most of

Table 1. Primers for detection of *P. larvae* spore DNA, targeted genes and length of expected PCR products

Primers	Gene	Sequence of primers	Length, bp
1)	16S rRNA	5′-AAGTCGAGCGGACCTTGTGTTTC-3′	973
2)		5′-TCTATCTCAAACCGGTCAGAGG-3′	
3) F3	MlP	5′-CGGGCAGCAAATCGTATTCAG-3′	273
4) B1		5′-CCATAAAGTGTTGGGTCCTCTAAGG-3′	
5) F6	16S rRNA	5′-GCACTGGAAACTGGGAGACTTG-3′	665
6) B11		5′-CGGCTTTTGAGGATTGGCTC-3′	
7) PL5	16S rRNA	5′-CGA GCG GAC CTT GTG TTT CC-3′	700
8) PL4		5′-TCA GTT ATA GGC CAG AAA GC-3′	
9) AF 6f	16S rRNA	5′-GCA AGT CGA GCG GAC CTT GT-3′	237
10) AF 7r		5′-GCA TCG TCG CCT TGG TAA GC-3′	

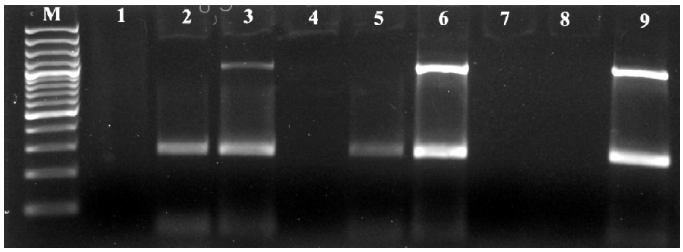

Fig. 1. Multiplex PCR detection of *P. larvae* spores in honey with primers 1–2/3–4 with diluted and undiluted DNA in three reaction mixtures. M: molecular marker (100–3000 bp), Fermentas; lane 1: honey sample (a), diluted 1:2 DNA 2 µL; lane 2: honey sample (b), diluted 1:2 DNA, 2 µL); lanes 3, 6, 9: positive control (*P. larvae* NBIMCC 8478); lane 4: honey sample (a), diluted 1:2 DNA 4 µL; lane 5: honey sample (b), diluted 1:2 DNA, 4 µL; lane 7: honey sample (a), undiluted DNA, 4 µL; lane 8: honey sample (b), undiluted, 4 µL.

the targeted gene fragments in honeys containing $\geq 10^2$ cfu/g. Amplification product was not generated only with primers 1–2 tested in multiplex PCR in combination with primers 3–4 (Fig. 1). The reaction was run with undiluted and diluted DNA in different ratios for determination of the influence of reaction inhibitors when working with honey. Single reaction mixtures were performed with the rest of the primers.

The electrophoregram for determining the sensitivity of PCR reaction with different primer pairs is presented on Fig. 2. The lowest sensitivity of the reaction is observed with primers 3–4 (10^{-1}), followed by primers 5–6 (10^{-2}), as with primers 7–8 and 9–10 the highest dilution of 10^{-3} was achieved.

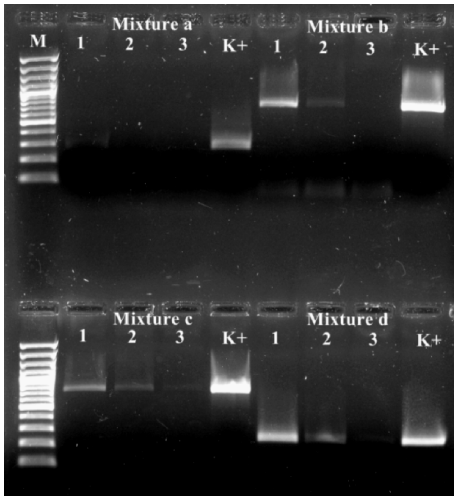

Fig. 2. Sensitivity of PCR reaction for detection of *P. larvae* spore DNA in mixtures with different primer pairs. M: molecular marker (100–3000 bp); *mixture a* contains primers 3–4; *mixture b:* primers 5–6; *mixture c:* primers 7-8; *mixture d:* 9–10; 1: DNA diluted 10^{-1}; 2: DNA diluted 10^{-2}; 3: DNA diluted 10^{-3}; K+ positive control (*P. larvae* NBIMCC 8478).

DISCUSSION

The timely detection of American foulbrood is essential to stop its spread in the affected apiary and adjacent apiaries. The identification is rendered difficult by potential contamination of samples with other spore-forming bacteria (Lindström & Fries, 2005; Bzdil, 2007). More rapidly replicating contaminating microflora impedes the isolation of *P. larvae* pure culture. On the other hand, there is no culture medium, highly selective for *P. larvae*. The known semi-selective media inhibit only some members of the genera *Bacillus*, *Brevibacillus*, *Paenibacillus* and *Vir-*

gibacillus (Schuch *et al.,* 2001), as confirmed by our experiments with trypticase soy blood agar supplemented with 20 μg/mL nalidixic acid. In cases of co-infection with European and American foulbrood, the presence of rapidly growing *Paenibacillus alvei*, eventually together with *Bacillus laterosporus*, impede the isolation of *P. larvae* in pure culture through conventional techniques (Abd Al-Fattah *et al.,* 2010; Rusenova *et al.,* 2013).

That is why, in previous experiments of ours, a conventional multiplex PCR for detection of *P. larvae* in isolated bacterial colonies and directly in putrid masses was developed for fast and accurate diagnostics of American foulbrood (Rusenova *et al.,* 2013). According to some researchers, honey is a main reservoir for spores of the bee family (De Graaf *et al.,* 2001; Fries & Camazine, 2001) and is important for the epidemiology of the disease due to its role in foulbrood spread. The analysis of honey for *P. larvae* spores is an element of national programmes for American foulbrood control in Denmark, Germany, Finland, Estonia, Australia (Hansen & Brodsgaard, 2003). The artificial swarm method, used worldwide and in our country for American foulbrood control consists in moving fee families, whose honey stores contain spores of American foulbrood agent. Therefore, the precise investigation of honeys for *P. larvae* spores is of particular significance for adequate control of the disease.

The present study attempted the isolation of spore DNA from honey of clinically affected and healthy bee families with subsequent conventional PCR detection, which would reduce the time of honey samples analysis. As *P. larvae* spores are known to be protected by seven layers, and those of *Bacillus spp*. – by 4–5 layers (Poppinga *et al.,* 2012), DNA extraction from spores was performed with a technique ensuring decoating of the spore with subsequent denaturation of proteins and destruction of spore wall and the cortex (peptidoglycan layer). Reviewing the literature, we utilised the protocol described by D'Alessandro *et al.* (2007), with a modification consisting in increased number of washings of spores with PBS aimed at better removal of PCR reaction inhibitors. Nevertheless, amplification products were obtained only when diluted DNA was used as seen from Fig. 1, in line with other PCR investigations in bee honey and bees (Piccini *et al.,* 2002; D'Alessandro *et al.,* 2007). DNA extraction from spores with chemical means is advantageous to the freeze-thaw method, as the latter does not detect spores in honey, probably because of DNA damage in the freeze and thaw stages (Bakonyi *et al.,* 2003; D'Alessandro *et al.,* 2007).

The results from the present study showed 70% sensitivity of the PCR in honey (14 out of 20 samples) with spore content $\geq 10^2$ cfu/g honey. Positive samples originated from the five diseased families and 2 of clinically healthy families. In positive samples, amplification of fragments from the metalloprotease and 16S rRNA *P. larvae* genes was achieved using primer pairs 3–4 and 5–6, 7–8 and 9–10 respectively with expected size of 278, 665, 700 and 237 bp. Amplification products were not generated with primers 1–2, flanking a 973 bp fragment of the bacterial 16S rRNA (Govan *et al.,* 1999), which was confirmed in earlier studies (Bakonyi *et al.,* 2003). The failed amplification of ribosomal gene segment with a size of 973 bp could be attributed to its higher molecular mass and impaired integrity during the isolation of spore DNA. For protocol sensitivity evaluation, pro-

ducts were generated with DNA dilutions from 10^{-1} to 10^{-3}. A similar sensitivity of the reaction for presence of *P. larvae* spores was reported in other studies. When analysing artificially contaminated honey by conventional PCR, Piccini *et al.* (2002) detected up to a 10^{-3} dilution of DNA from a spore suspension containing 170 cfu/mL. Bakonyi *et al.* (2003) tested 23 honey samples containing *P. larvae* spores in conventional PCR and detected 18 as positive. The samples negative in the PCR tests contained 3.2 to 33.8 cfu as per the culture method. The reported sensitivity of the reaction of 78.3% was similar to that in the present study (14/20; 70%). Among the tested 91 honey samples, Bassi *et al.* (2010) established 34 samples positive in both conventional PCR and culture method, 15 PCR-positive and culture-negative, as well as 14 PCR-negative and culture-positive. The PCR-negative samples in the studies of Bassi *et al.* (2010) had a spore load between 1 and 7 cfu/g.

The direct PCR detection of *P. larvae* in honey is this study yielded a negative result in three of bee families without signs of American foulbrood and spore contamination levels between 10–46 cfu/g.

CONCLUSION

False negative results in conventional PCR tests of bee honeys with low contamination levels could have a negative impact on measures for disease control and could permit spread of spores out of the affected bee family. That is why for adequate control of American foulbrood through detection of *P. larvae* spores in honey samples, we recommend isolation of the pathogen on suitable agar medium followed by PCR identification in bacterial colonies. Despite the longer period required when culturing was combined with PCR, the detection rate of *P. larvae* spores was 100% whereas the sensitivity of direct PCR detection of spores in honey was below 80%. All the same, if conventional PCR detection of honey is selected, we recommend the use of primer pairs 5–6, 7–8 or 9–10, which generate specific amplicons with DNA dilutions from 10^{-2} to 10^{-3}. We believe that the results of the present study would be helpful for American foulbrood control.

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