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Scientific note

A scientific note on the detection of spores of *Paenibacillus larvae* in naturally and artificially contaminated honey: comparison of cultural and molecular methods*

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***Paenibacillus larvae* / spore / honey / PCR / bacterial culture**

The detection of *Paenibacillus larvae* spores in honey produced by infected colonies enables an early identification of American foulbrood (Ritter, 2003). Bacterial culture with colony counting is the reference method for *P. larvae* spores assessment in honey (OIE, 2008), but it is time consuming and the overgrowth of other *Bacilli* can confuse the results.

Kilwinski et al. (2004) developed a three-reaction PCR protocol with high specificity towards *P. larvae* for accurate species identification. Given the specificity of that protocol, in our study we assessed the sensitivity of one of those reactions as a faster alternative to culture for the detection of *P. larvae* spores in honey samples. The reaction was chosen for its superior efficiency compared to the others (unpublished data).

The analytical sensitivity of both PCR and bacterial culture was determined. Then, PCR was compared to culture to determine its ability to discriminate non-contaminated from contaminated samples with different loads of spores.

One hundred and two samples were tested. They included 91 honeys from apicultural production, 4 artificially-contaminated honey samples containing approximately 10 000, 1000, 100 and 10 spores of *P. larvae* per gram, 1 spore-free honey, 6 water suspensions of *P. larvae* spores ranging from 10^4 to 10^{-1} spores/mL. The spores for water suspensions and artificially-contaminated honeys were harvested from dead larvae and counted in a Bürker chamber.

Samples were pre-treated for culture and PCR as follows: five grams of each honey sample were diluted with 5 mL of sterile distilled water by vigorous shaking (obtaining a final volume of 8.8 mL) and centrifuged at $3500 \times g$ for 45 min. The supernatant was eliminated leaving 2 mL in the tube and the pellet was re-suspended in this volume by vigorous shaking for 1 min. The re-suspended sample was heated at 85–90 °C for 15 min in a water bath (Alippi et al., 2004). Water suspensions were subjected to heat treatment without previous dilution and centrifugation. Artificially-contaminated honeys were diluted with 5 mL of sterile distilled water (obtaining a final volume of 8.8 mL) and subjected to heat treatment without previous centrifugation.

Cultural method

Four hundred microliters of each pre-treated sample were plated onto 4 plates (100 μL/plate) of MYPGP agar (Dingmann and Stahly, 1983), with 3 mg/L of nalidixid acid (Hornitzky and Clark, 1991). The plates were incubated at 37 °C in 10% CO_2 and examined after 3 and 8 days. Five suspected colonies per plate were tested for catalase reaction. Catalase-negative colonies were Gram stained and Gram-positive rods were directly submitted to PCR for species confirmation (DNA was extracted by heat treatment).

PCR

Following pre-treatment, the samples (1.5 mL for honeys, 4 mL for water suspensions, 8 mL for

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Table I. Evaluation of the analytical sensitivity for artificially-contaminated samples.

Artificially-contaminated honey samples			Water suspensions		
Added spores /g	Result		Added spores /mL	Result	
	PCR	Culture (CFU/g)*		PCR	Culture (CFU/mL)*
10 000	Pos.	Pos. (188)	10 000	Pos.	Pos. (293)
1000	Pos.	Pos. (33)	1000	Pos.	Pos. (76)
100	Pos.	Pos. (8)	100	Pos.	Pos. (6)
10	Neg.	Neg.	10	Pos.	Neg.
0	Neg.	Neg.	1	Neg.	Neg.
			0.1	Neg.	Neg.

* In brackets the mean (rounded up) of two independent experiments performed in triplicate ($N = 6$ for each level of contamination).

artificially-contaminated honeys) were centrifuged at 3500 × g for 45 min and the pellet re-suspended with 200 μL of Lysozyme solution followed by proteinase K treatment as previously reported (Bakonyi et al., 2003). DNA was purified by NucleoSpin tissue minikit (Macherey – Nagel) and eluted in 100 μL of elution buffer. PCR was performed in a reaction volume of 25 μL (with 5 μL of template DNA), according to Fast Start Taq kit (Roche), with 0.5 μM of primers PLL-16S F6 and PLL-16S B11 (Kilwinski et al., 2004).

The amplification protocol comprised 5 min at 95 °C, followed by 40 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 7 min.

Ten microliters of amplification product were electrophoresed on 1.8% agarose gel followed by staining in ethidium bromide (1 μg/mL) and visualization by UV transillumination.

Analytical sensitivity was determined in two separate experiments carried out in triplicate. Results are summarised in Table I.

In artificially-contaminated honeys the limit level of 100 spores/g was detected by culture and PCR. At this level of contamination, culture revealed 8 CFU/g. Lauro et al. (2003) reported a similar sensitivity (9 CFU/mL) with a nested PCR preceded by an incubation step in MYPGP broth. Conversely, our protocol is based on a direct PCR with a reduced probability of false-positives and without pre-incubation, to increase simplicity.

In water suspensions of spores the detection limit of the cultural method was 100 spores/mL, while PCR was able to detect 10 spore/mL, corresponding to the template DNA from 2 spores per reaction. This difference was probably due to the limited germination capacity of spores on culture media (Nordström and Fries, 1995). In this study we observed germination rates between 1.9% and 8% in artificially-contaminated honeys and between 2.9% and 7.6% in water suspensions. These results are consistent with those of Forsgren et al. (2008). The superior sensitivity of PCR for water suspensions compared to honeys could be due to PCR inhibitors or less effective DNA extraction from honey. Piccini et al. (2002) have already reported strong PCR inhibition by honey. These authors needed 10^{-1} dilution of the DNA extracted by a simple microwave treatment to have amplification, obtaining a sensitivity of 170 CFU/mL. The extraction procedure that we used significantly reduced inhibition with only 1 Log difference in sensitivity between water suspensions and honeys, without DNA dilution.

Among the naturally-occurring honey samples, 34 were PCR and culture positive, 28 were PCR and culture negative, 15 were PCR positive and culture negative and 14 were PCR negative and culture positive. Culture positive samples ranged from 1 to 1460 CFU/g. Among them, the 14 PCR-negative samples had low CFU/g counts (between 1 and 7).

In conclusion, these results indicate that the PCR protocol employed is as sensitive as culture with MYPGP for *P. larvae* spores detection in honey, but it gives results in 24 hours instead of 8 days. Furthermore, the combination of DNA extraction and amplification protocol that we propose is simpler and less prone to false-positives than the previously proposed nested PCR by Lauro et al. (2003).

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Note scientifique sur la recherche de spores de *Paenibacillus larvae* dans des échantillons de miel naturellement et artificiellement contaminés : comparaison entre méthodes culturales et moléculaires.

Eine wissenschaftliche Notiz über den Nachweis von Sporen von *Paenibacillus larvae* in natürlich und künstlich kontaminierten Honigproben: Ein Vergleich von Kultivierungs- und molekularen Methoden.

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