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Note

Triplex real-time PCR method for the qualitative detection of European and American foulbrood in honeybee

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ABSTRACT

The bacteria *Melissococcus plutonius* and *Paenibacillus larvae*, causative agents of respectively European and American foulbrood, damage honeybee health worldwide. Here, we present a specific and sensitive qualitative triplex real-time PCR method to detect simultaneously those microbial agents and a honeybee gene, validated through a study involving 7 laboratories through Europe.

Short note:

American foulbrood (AFB) caused by *Paenibacillus larvae* and European foulbrood (EFB) induced by *Melissococcus plutonius* are both major honeybee (*Apis mellifera*) brood disease globally widespread (Ellis and Munn, 2005) and contribute to colony declines (Forsgren, 2010; Genersch, 2010). Both pathogens develop in the gut when ingested by the larvae at any stage for EFB (Bailey and Ball, 1991) or only within the first 72 h after hatching for AFB (Bamrick and Rothenbühler, 1961, Brodsgaard et al., 1998). Four AFB type, so called ERIC type have been described (Genersch et al., 2006). Only ERIC I and II are usually detected in the field (Genersch, 2010). AFB and EFB are notifiable diseases in many countries where the authorities usually destroy the colonies with clinical symptoms. Hence, foulbrood can lead to both serious economic loss for beekeepers and considerable efforts from veterinary authorities.

In Switzerland EFB prevalence dramatically increased the last decade where a first real-time PCR system has been published (Roetschi et al., 2008). Therefore early diagnostic is from utmost importance, using accurate, rapid, cheap and easy to handle detection tools, to prevent its spread. Here we present a qualitative real-time triplex PCR method for the detection of EFB, AFB (including ERIC type I to IV) and a specific gene of the honeybee host as PCR amplification control verified by a validation study involving 7 laboratories European Union wide. To our knowledge this is the first DNA-based method to detect all three listed target organisms simultaneously. Field samples were obtained by cutting out a section of brood, transported immediately to the laboratory and stored at −20 °C until further processing.

The DNA was extracted with the NucleoSpin Tissue kit from Macherey-Nagel according to the user manual with the following modifications: During the pre-lyse step, prior to the proteinase K, a lysozyme digestion (20 mg lysozyme/ml at 37 °C for 1 h) and after the Proteinase K digestion a RNAse A incubation (20 mg/ml at room temperature for 5 min) was added. DNA concentration in extracts was measured with the NanoDrop® ND-1000 spectrophotometer (NanoDrop, Thermo Fischer Scientific, USA).

For the primer-probe design, performed with the Beacon Designer 8.20, the following gene sequences were used: for *Melissococcus plutonius*: napA pseudogene, Na+/H+ antiporter, strain: ATCC 35311, Accession #: AB778538.1 (Arai et al., 2014), for *Paenibacillus larvae* the sequences of the amplicon of primer pair ERC0390F/ERC03906R, including gene tnp60 (Djukic et al., 2014). This region belongs to P. larvae DSM 25430, Accession #: CP003355.1, and for *Apis mellifera* mRNA for actin, partial cds, accession # AB023025.1 (Roetschi et al., 2008). All primers and probes were examinated for similarity using the NCBI BLAST tool. The results confirmed the theoretical specifity of the target regions.

Information on primer and probe sequences, the labelling fluorophores with its quenchers and the amplicons are listed in Table 1.

The PCR was set up in a 20 µl volume, with 2× Mastermix (SensiFast Probe No-ROX Kit from Bioline) containing 400 nM of each primer, 100 nM of each probe and 5 µl DNA-extract (1 ng/µl).

The amplifications were performed with the Rotor-Gene 6000, Qiagen (formerly Corbett Life Science). According to the Bioline recommendations the following cycling program was applied: 5 min at 95 °C and 45 cycles of 10 s at 95 °C and 30 s at 60 °C. The Rotor-Gene

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Table 1
Primer probe and amplicon information.

Target organism	Primer/Probe	Oligonucleotide sequence (5′-3′) and probe labelling	Amplicon length	Position of the amplicon[a]
M. plutonius	MP-F	GAC CTG TTT AGC TAT TAT CAC TA	92	890–981
	MP-R	CAC CTA CAA TGA ATG ATT CAT TC		
	MP-Probe	FAM – TCC GCC TAA GCT ACC ACC TAA GAA C - BHQ1		
P. larvae	PL-F	TAC GCT TTT CGA TTC TCT G	87	918′841–918′927
	PL-R	GTC TGT ACT GAA CCA AGT C		
	PL-Probe	Yakima Yellow – ATC TGC TTC CAC TTG TTC ACT CAC CA - BHQ1		
A. mellifera	AM-F	TCC AGA TGG TCA AGT AAT TAC	87	52–138
	AM-R	GCT TCC ATT CCT AAG AAG G		
	AM-Probe	ROX – TCC GTT GTC CCG AGG CTC TTT - BHQ2		

[a] The specified positions of the amplicons correspond to the sequences listed under the accession numbers mentioned in the text above. For *P. larvae* the listed position of the amplicon is on gene tnp60.

Table 2
Mixtures of EFB *M. plutonius*, AFB *P. larvae* and *Apis mellifera* with different concentrations.

70.0% AFB + 20.0% *A. mellifera* + 10.0% EFB
75.0% AFB + 20.0% *A. mellifera* + 5.0% EFB
80.0% AFB + 17.5% *A. mellifera* + 2.5% EFB
70.0% EFB + 20.0% *A. mellifera* + 10.0% AFB
75.0% EFB + 20.0% *A. mellifera* + 5.0% AFB
80.0% EFB + 17.5% *A. mellifera* + 2.5% AFB
70.0% *A. mellifera* + 15.0% EFB + 15.0% AFB
90.0% *A. mellifera* + 5.0% EFB + 5.0% AFB
95.0% *A. mellifera* + 2.5% EFB + 2.5% AFB

software version 2.3.2.49 was used to analyze the raw data.

Prior to a validation study the triplex real-time PCR was tested with DNA extracted from the following target organisms: *Melissococcus plutonius*, *Paenibacillus larvae* (ERIC I, II, III and IV) and *Apis mellifera*. All possible combinations in practice-oriented concentrations were tested (Table 2).

The limit of detection (LOD) for EFB and AFB was set at a quantification cycle Cq-value of 30 (corresponding to 0.001 ng/PCR or 456 copies number resp. 0.0001 ng/PCR or 20 copies) and for *Apis mellifera* at a Cq-value of 32 (corresponding to 0.1 ng/PCR or 380 copies). From the view of the practical relevance these LODs are considered as absolutely satisfactory. For each target organism and each concentration the Cq-value was always significantly above the LOD, meaning that even in a mixture of the two pathogens low contamination levels can be detected.

To test the fitness of the method an international validation study was organized. Thirteen blind samples (Table 3a), each in a tube labelled with a randomized number, containing approximately 100 µl extracted DNA (10 ng/ µl) together with 2 tubes containing the lyophilized primers and probes and a detailed working instruction was shipped to 7 laboratories.

The fitness of this method was assessed by computation of the accuracy (AC), sensitivity (SE) and specifity (SP) for each single laboratory and target organism and calculated as follows:

Accuracy (AC) is the fraction of correct positive and negative

Table 3
a) List of the samples with its expected results used for the validation study. b) Validation study results of the accuracy, sensitivity and specificity. N/A: no answer.

a

Sample	DNA extract	expected qPCR result EFB	expected qPCR result AFB	expected qPCR result bee
1	*E. coli* DH5α	Negative	Positive[a]	Negative
2	*M. plutonius* ATCC 35311 (Reference)	Positive	Negative	Negative
2	*M. plutonius* 49.3 (CH)	Positive	Negative	Negative
4	*M. plutonius* 27.1 (FR)	Positive	Negative	Negative
5	*P. larvae* DSM 7030 (ERIC I, Reference)	Negative	Positive	Negative
6	*P. larvae* BK 199–13 (ERIC II, GER)	Negative	Positive	Negative
7	*P. larvae* CCUG 48972 (ERIC II, SE)	Negative	Positive	Negative
8	10% *M. plutonius* ATCC 35311 & 90% *P. larvae* DSM 7030	Positive	Positive	Negative
9	90% *M. plutonius* ATCC 35311 & 10% *P. larvae* DSM 7030	Positive	Positive	Negative
10	90% *A. mellifera* ATCC 35311 & 10% *P. larvae DSM 7030*	Negative	Positive	Positive
11	90% *A. mellifera* & 10% *M. plutonius ATCC 35311*	Positive	Negative	Positive
12	*A. mellifera*	Negative	Negative	Positive
13	80% *A. mellifera* & 10% *P. larvae DSM 7030* & 10% *M. plutonius ATCC 35311*	Positive	Positive	Positive

b

	Melissococcus plutonius			*Paenibacillus larvae*			*Apis mellifera*		
Lab	AC	SE	SP	AC	SE	SP	AC	SE	SP
2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3	1.00	1.00	1.00	1.00	1.00	1.00	0.77	0.25	1.00
4	1.00	1.00	1.00	0.92	0.88	1.00	1.00	1.00	1.00
5	1.00	1.00	1.00	0.92	1.00	0.80	1.00	1.00	1.00
6	1.00	1.00	1.00	1.00	1.00	1.00	N/A	N/A	N/A
7	1.00	1.00	1.00	1.00	1.00	1.00	0.46	0.50	0.44
9	0.85	0.71	1.00	1.00	1.00	1.00	N/A	N/A	N/A

[a] Contaminated with *P. larvae*.

results; it is calculated by the following equation:

AC is: $\frac{PA + NA}{PA + ND + PD + NA}$.

Sensitivity (SE) is the ability of classifying positive results as positive, it is calculated as follows:

SE is: $\frac{PA}{PA + ND}$

Specificity (SP) is the ability of classifying negative results as negative, it is calculated as follows:

SP is: $\frac{NA}{PD + NA}$

with:

PA: positive agreement (i.e. number of times detection was done when expected)

NA: negative agreement (i.e. number of times there was no detection when expected)

PD: positive deviation (i.e. number of times detection was done even though detection was not expected)

ND: negative deviation (i.e. number of times there was no detection even though detection was expected)

The results of the statistic parameters AC, SE and SP reported from each laboratory with all target organisms are listed in Table 3b. All 7 participating laboratories could successfully detect EFB and AFB in the 13 samples (Table 3a).

For *M. plutonius* only one laboratory reported 2 false negative results of a total of 49 samples.

For *P. larvae* 5 laboratories reported results with no errors. One laboratory reported 1 false negative of a total of 56 positive samples and another 1 false positive result of a total of 35 negative samples.

Due to the fact that the probe for the *A. mellifera* PCR-system was labelled with ROX, 2 laboratories were not able to work with this probe: one laboratory had to use a mastermix containing ROX as fluorophore for the instrument calibration, the second had a PCR machine with two channels only.

From the remaining 5 laboratories 3 reported results with no errors for *A. mellifera*, one with false positive and one with false negative and false positive results (Table 3b). Taking into account that the two laboratories reporting false results did not use a mastermix optimized for triplex real-time PCR (compared to the 3 laboratories submitting no errors for *A. mellifera*) these results are fully plausible.

Based on the results of the validation study this method can be considered as fit for purpose.

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