Protocol instruction: Identification of *Paenibacillus larvae*, agent of American foulbrood, using PCR (in-house method)

The original is kept at the research unit

Contents

Effective date: 01/11/2013
Protocol established according to the ANA-I1.MOA.19_Rev02

This document may not be copied without the agreement of the ANSES Sophia Antipolis laboratory
Tel.: +33 (0)4-92-94-37-00 Fax.: +33 (0)4-92-94-37-01

1 PURPOSE AND SCOPE

This instruction describes the identification of the main agent of American foulbrood (AFB), *Paenibacillus larvae*, in brood samples (larvae) using polymerase chain reaction (PCR). This protocol is based on the detection of a 16S rRNA sequence specific to *Paenibacillus larvae*.
The initial diagnosis of AFB is made during a bacterioscopic examination after Gram staining. Identification of the main agent of this disease is then confirmed using PCR.

AFB is a notifiable disease.

2 CONTENT

2.1 Principle

The technique used is based on the PCR protocol described by Dobbelaere *et al*. (2001) and included in the OIE manual (2008).
The pair of primers used (AFB-F & AFB-R) amplifies a PCR product of 1096 bp (Dobbelaere *et al*., 2001).

The amplification technique is divided into four steps:

1. Preparation of honeybee larvae samples
2. Extraction of genomic DNA (not fully described in this instruction)
3. Amplification of target DNA and analysis of PCR products
4. Interpretation and validation of the results

2.2 Preparation of honeybee larvae samples

- From a diseased brood, take two or three larvae in sealed cells that show characteristic clinical signs
- Put the larvae in a 1.5 ml microcentrifuge tube containing 500 µl of ultra pure water.
- Crush the larvae with a disposable pestle for a 1.5 ml tube and homogenise the sample by vortexing. These tubes are used for bacterioscopic analyses in order to identify *P. larvae* spores as well as for DNA extraction. Store the tubes containing larval suspensions in the freezer at a temperature of ≤ -16°C, if the two are not done in a row.

2.3 Genomic DNA extraction

Extract genomic DNA from suspected specimens with chosen relevant method ensuring quality of nucleic acids extracted

Note: The detection and identification of insect or honeybee pathogens by PCR is wholly dependent upon the availability of 'clean' nucleic acid mixtures to act as a template for the reactions (OIE *Terrestrial Manual*, Guideline 3.2, 2012). This stage is critical because if the target material has not been purified of contaminants in

This document may not be copied without the agreement of the ANSES Sophia Antipolis laboratory
Tel.: +33 (0)4-92-94-37-00 Fax.: +33 (0)4-92-94-37-01

the clinical sample, the assay stage is compromised and may yield false results (OIE *Terrestrial Manual*, Chapter 1.1.5, 2012).
There are a number of specialised methods for particular types of samples and tissues, some of which are now commercially available either as manual or automated systems for robotic workstations.

Note: Regardless the DNA extraction method used, the EU RL for honeybee health recommends to add a positive extraction control, to ensure the effectiveness of the extraction, and a negative extraction control (replacing the sample with ultra pure water) to ensure absence of contamination (when PCR is used for diagnosis, a great deal of care is required to avoid contamination of the samples because the exquisite sensitivity of the technique can easily lead to false-positive results (OIE *Terrestrial Manual*, Chapter 1.1.5, 2012).
The EU RL recommends to estimate the sensitivity (limit of detection) of the method in the user's laboratory condition (following an appropriate validation scheme) to ensure that the method is able to properly detect the targeted insect or pathogen (OIE *Terrestrial Manual*, Chapter 1.1.5. 2012). The positive extraction control can be aliquoted in a small volume and then stored at a temperature of ≤ -16°C in the freezer. Alternatively, positive extraction control homogenate can be provided by the EU RL for honey bee health.

Before DNA extraction, it is recommended to treat the samples with proteinase K, if this step is not included in the DNA extraction method.

2.4 DNA amplification and analysis of PCR products

DNA is amplified by PCR with specific primers. The reaction is based on the succession of temperature cycles, each cycle containing three steps: denaturation, annealing and elongation. Products obtained at the end of each cycle are then matrix for the following cycle. Thus, the amplification is exponential and allows to obtain 2^n copies of DNA (n = number of cycles). Primers and size of PCR products are specific to each sequence, so the primer annealing temperature and the time of annealing and elongation change according to the sequence to be amplified.

2.4.1 Specific points

The specific primers used for typing *P. larvae* and the expected PCR product size are as follows:

Primer	Sequence	PCR product size (bp)	Specificity
AFB-F	5' - CTT GTG TTT CTT TCG GGA GAC GCC A - 3'	1096	*Paenibacillus larvae*, AFB
AFB-R	5' - TCT TAG AGT GCC CAC CTC TGC G - 3'		

- The sample volume used is 5 µl.
- An AFB PCR positive control must be added (Clone H5 plasmid positive control diluted to the concentration of the PCR limit of detection (LOD_{PCR}) multiplied by 10). Add 5 µl of the clone H5 in the corresponding tube.
- A PCR negative control must be added (5 µl of pure distilled water instead of 5 µl of sample).

2.4.2 Preparation of the reaction mix

This document may not be copied without the agreement of the ANSES Sophia Antipolis laboratory
Tel.: +33 (0)4-92-94-37-00 Fax.: +33 (0)4-92-94-37-01

Amplification is performed in a 25 µl final volume (20 µl of mix + 5 µl of DNA).

	Final concentration	For example:	Volume for 1 tube (µl)
H20	/	H20	13.8
Taq polymerase buffer	1X	Taq polymerase buffer (10X)	2.5
MgCl2	3 mM	MgCl2 (50 mM)	1.5
dNTP	400 µM	dNTP (10 mM)	1
Primer 1	400 nM	Primer 1 (20 µM)	0.5
Primer 2	400 nM	Primer 2 (20 µM)	0.5
Taq Pol	1 unit / 25 µl	Taq Pol (5U/ µl)	0.2
			---
			20 µl

Homogenise the mix with a pipette or a vortex and centrifuge quickly in a table-top centrifuge.
Distribute 20 µl of mix per tube.
Add 5 µl of sample DNA, or 5 µl of positive control, or 5 µl of water in the corresponding PCR tubes.

2.4.3 PCR programme

The specific characteristics of this programme have been underlined:

- DNA denaturation 94°C for 2 minutes
-
- 30 cycles: Denaturation at 94°C for 30 seconds; Primer annealing at 55°C for 30 seconds; Elongation at 72°C for 1 minute
- Final elongation 72°C for 5 minutes
- Final hold at 10°C

When the run is finished, place the tubes at 5°C +/- 3°C, before the analysis on an agarose gel.

2.5 Analysis of the PCR products by electrophoresis on agarose gel

Electrophoresis technique is based on the separation of negatively charged nucleic acids, under the effect of an electric field. This separation is performed into agarose gel matrix: the smallest molecules move faster and migrate farther than the largest ones. Estimation of the size fragment is done comparing with a DNA-ladder, which migrates simultaneously in another well of the same gel.
The staining method used is the revelation with ethidium bromide (EtBr). EtBr is an intercalating agent commonly used in molecular biology laboratories to observe nucleic acids. When exposed to ultraviolet it fluoresces. This fluorescence is 20 times higher when EtBr is linked to DNA molecules.

2.5.1 Preparation of the agarose gel

This document may not be copied without the agreement of the ANSES Sophia Antipolis laboratory
Tel.: +33 (0)4-92-94-37-00 Fax.: +33 (0)4-92-94-37-01

- It is recommended to prepare the agarose gel with TAE 1X.
- A gel with 1.2 % (w/v) of agarose can be prepared to observe PCR products. For example weight 1.2 g of agarose for 100 ml of TAE 1X.According to the size of the PCR products agarose percentage can be higher or lower (high concentrations of agarose will be used to observe small PCR products).
- Heat the TAE-agarose in a microwave until obtaining a transparente liquid.
- Cool a few minutes before pouring the solution into a gel rack, containing the comb.
- Wait until the gel has solidified.
- Remove the comb and place the gel into the electrophoresis tank.
- Pour TAE 1X to cover the gel.

2.5.2 Preparation and loading of the samples

- It is recommended to migrate 10 µl of the PCR products.
  Loading buffer must be added to the sample before migration.
- Load the samples into the wells.
- Load a DNA-ladder.
- Run the gel.

2.5.3 Gel staining with EtBr

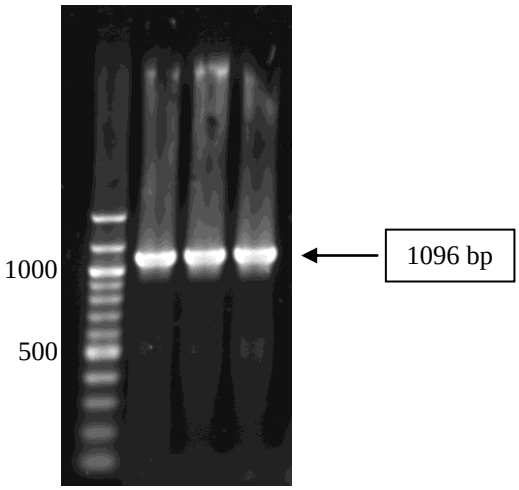
EtBr is a potent mutagen. It is strongly recommended to wear nitrile gloves, a lab coat and protective glasses.

- Put the gel in a 0.5µg/ml EtBr solution, for at least 20 minutes. The duration can be increased if the gel contains a high percentage of agarose, if it is too thick or if the EtBr solution is too old.
- Observe the gel with ultraviolet.
- Take a picture of the gel.

2.6 Interpretation and validation of results

When using this PCR to identify *P. larvae*, interpretation of the results are based on the presence or absence of the amplified product (PCR product size is 1096 bp).
Photo: Validation of the presence of the main agent of AFB, *P. larvae*, in the analysed samples compared with a 100 bp molecular size marker.

This document may not be copied without the agreement of the ANSES Sophia Antipolis laboratory
Tel.: +33 (0)4-92-94-37-00 Fax.: +33 (0)4-92-94-37-01



A PCR result is only considered valid if:

- The positive extraction and positive PCR controls are both positive,
- The negative extraction and negative PCR controls are both negative.

Moreover, if there is a negative identification result, the absence of PCR inhibitors in the analysed extract must be checked, as this could lead to a "false negative" result. A PCR targeting the β-actin gene is performed to confirm the amplification of the *Apis mellifera*-specific β-actin gene, which thus acts as a non-target endogenous positive control (see "Test for the presence of the β-actin gene on honeybee samples using PCR"). If the presence of the β-actin gene is confirmed, the negative result for the analysed sample is confirmed. Otherwise, the extraction must be repeated for the sample in question.

A negative identification result by PCR leads to the conclusion "pathogen untyped".

3 REFERENCES

Dobbelaere W., De Graaf D.C., Peeters J.E and Jacobs F.J. (2001) - Development of a fast and reliable diagnostic method for American foulbrood disease (Paenibacillus larvae subsp. larvae) using a 16S rRNA gene based PCR - Apidologie, **32**, pp. 363–370.

Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2008 edition (World Organisation for Animal Health - 12, rue de Prony - 75017 Paris - France) pp 395 –404

This document may not be copied without the agreement of the ANSES Sophia Antipolis laboratory
Tel.: +33 (0)4-92-94-37-00 Fax.: +33 (0)4-92-94-37-01