The original is kept at the research unit

Contents

Protocol established according to the ANA-I1.MOA.11

1 PURPOSE AND SCOPE

This instruction describes the identification of *Nosema* spp. *(Nosema apis* or *Nosema ceranae)* using polymerase chain reaction (PCR), following diagnosis by optical microscopy. This protocol is based on the detection of a 16S rRNA sequence of the microsporidia, specific to the species, from homogenates prepared according to the OIE manual.
The initial diagnosis of nosemosis is made during a bacterioscopic examination. Identification of the agent(s) of this disease is then confirmed using PCR.

2 CONTENT

2.1 Principle

The technique used is based on a duplex PCR described by Martin-Hernandez *et al.* (2007) and included in the OIE manual (2008).

However, because problems were encountered in the EU RL to detect *N. apis* when *N. ceranae* is abundant, two monoplex PCRs are carried out to avoid non-detection of *N. apis* in the event of co-infection.

Two pairs of primers are used, each one specific to the target species.
The technique for identifying *Nosema* species by amplification can be divided into four steps:

1. Preparation of honeybee homogenates (not described in this instruction)
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 spore homogenates

Homogenates can be prepared according to the OIE method (2008). Samples are analyzed with an optical microscope. Sample positive for nosemosis are further used for PCR analyses.

2.3 Extraction of genomic DNA

2.3.1 Sample preparation

Positive samples identified from the nosemosis diagnosis using an optical microscopy method are stored at around -20°C until subsequent processing.
The spore suspension is prepared in a 50 ml tube, centrifuged for 6 minutes at 800 g.
The supernatant is removed and the pellet is resuspended in 1.5 ml of ultra pure water, and then placed on ice. A volume of 80 µl of the suspension will be used for extracting *Nosema* genomic DNA. The remaining solution is stored in a freezer at about -20°C.

2.3.2 Extraction of genomic DNA

Note: Whatever the DNA extraction method used, it is recommended to add two positive extraction controls (if available), a *N. apis* control and a *N. ceranae* control, as well as a negative extraction control (replacing the sample with ultrapure water).

- To facilitate the pipetting, the sample should be centrifuged rapidly to pellet the suspended debris.
- Treat the samples with proteinase K before DNA extraction. It is recommended to treat 80 µl of sample, 1.30 hours at 55°C. Vortex occasionally. Visually check that the lysis is complete.
- Extract DNA with the chosen 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.

For each sample, two PCRs are carried out to identify *N. apis* and *N. ceranae* separately. Prepare two different series of tubes (one for each PCR reaction mix).

2.4.1 Specific points

The specific primers used for typing *N. apis* and *N. ceranae* are as follows:

Primer	Sequence [a]	PCR product size (bp)	Specificity
218 MITOC FOR	5' - CGGCGACGATGTGATATGAAAATATTAA - 3'	218–219 [b]	*N. ceranae*
218 MITOC REV	5' - CCCGGTCATTCTCAAACAAAAAACCG - 3'		
321 APIS FOR	5' - GGGGGCATGTCTTTGACGTACTATGTA - 3'	321	*N. apis*
321 APIS REV	5' - GGGGGGCGTTTAAAATGTGAAACAACTATG - 3'		

According to Martin-Hernandez *et al.* (2007) and the OIE Manual 2008:
(a) the CG bases added to the primers are underlined.
(b) there is a difference of one base pair in the size of PCR products which depends on the sequences of *N. ceranae* available in the GenBank database (http://www.ncbi.nlm.nih.gov).

- The sample volume used is 5 µl.
- A PCR positive control must be added in each monoplex PCR (5µl):
  - *N. apis* PCR: *N. apis* clone K3a plasmid positive control diluted to the concentration of the PCR limit of detection (LOD_{PCR}) multiplied by 10.
  - *N. ceranae* PCR*: N. ceranae* clone L2 plasmid positive control diluted to the concentration of the PCR limit of detection (LOD_{PCR}) multiplied by 10.
- 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

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

	Final concentration
H20	/
Taq polymerase buffer	1X
MgCl2	3 mM
dNTP	400 µM
Primer 1	400 nM
Primer 2	400 nM
Taq Pol	1 unit / 25 µl

For example:

	Volume for 1 tube (µl)
H20	13.8
Taq polymerase buffer (10X)	2.5
MgCl2 (50 mM)	1.5
dNTP (10 mM)	1
Primer 1 (20 µM)	0.5
Primer 2 (20 µM)	0.5
Taq Pol (5U/ µl)	0.2
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	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 the programme have been underlined:

- DNA denaturation — 94°C for 2 minutes
- 35 cycles:
  - Denaturation at 94°C for 30 seconds
  - Primer annealing at 62°C for 30 seconds
  - Elongation at 72°C for 30 seconds
- Final elongation — 72°C for 7 minutes
- Final hold — 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 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 can be 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.
Other techniques are available to estimate the size of the amplified products such as capillary electrophoresis or microfluidic electrophoresis. The protocol described below is given as an example.

2.5.1 Preparation of the agarose gel

- 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 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

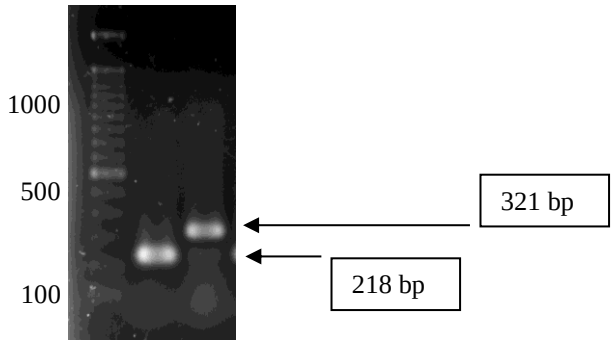
EtBr is a potent mutagen. It is strongly recommended to wear nitrile gloves, a lab coat and protective glasses.

- 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 these uniplex PCRs to identify the *Nosema* species, interpretation of the results is based on detection of target DNA of both species *(N. apis* and *N. ceranae)* depending on the size of the PCR products generated. For a sample typed as *N. apis*, the size of the PCR product will be 321 base pairs. For a sample typed as *N. ceranae*, the size of the PCR product generated will be 218 base pairs (or 219 bp depending on the sequence).

Photo: Validation of discrimination between *N. ceranae* (lane 1) and *N. apis* (lane 2) compared with a 100 bp molecular size marker.



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 neither pathogen has been positively identified in the same sample in which *Nosema* spores were observed, the absence of PCR inhibitors in the 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 the document ***ANA-I1.MOA.3000*** "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

Carletto, J., Blanchard, P., Gauthier, A., Schurr, F., Chauzat, M.P., and Ribière, M. (2013). Improving molecular discrimination of *Nosema apis* and *Nosema ceranae*. J. Invertebr. Pathol. 113, 52-55.

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