Analytical method for animal health

REFERENCE: ANSES/SOP/ANA-I1.MOA.0900 - Version 02

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Nosemosis diagnosis: identification and quantification of *Nosema* spp. by microscopic examination

Sophia-Antipolis Laboratory
National Reference Laboratory – Bee Health
European Union Reference Laboratory –Bee Health

History of the method

A method may be updated to take changes into account.

A change is considered major when it relates to key parts or to the substance of the method, which when taken into account is likely to significantly improve the scope or result of the analytical method. A major change generally leads to significant adaptations. The amended method is then revalidated.

A change is considered minor if it provides useful or practical clarifications, reformulates the words to make them clearer or more precise, and corrects minor errors. The performance characteristics of the improved method are unchanged; it is not revalidated.

The table below summarises the version history of this method.

Version	Nature of the changes	Date	Main changes
V01	Creation	10/05/2017	Initial version: protocol established according to the ANA-I1.MOA.09 (revision 05)
V02	Minor revision	26/05/2020	▪ Editorial updates. ▪ Precisions: - Size of *Nosema* spores (better taking into account the extreme dimensions of *N. ceranae* spores): § 5, § 8.3 - Better consideration of "inconclusive" cases and the importance of the quality of the sample recevied: § 7.1, § 8.3, § 9.2 - Cases where rare spores are detected outside the counting area of the counting chamber: § 8.2 and § 9.2 - Guidelines for presenting analytical results and expressing interpretations and opinions: § 9.2. and § 9.3. - Addition of bibliographical references and reference documents: § 2 and “References”

Foreword

This method has been optimised by:

ANSES - Sophia-Antipolis Laboratory

National Reference Laboratory for Bee Health

European Reference Laboratory for Bee Health

OIE Reference Laboratory for Honey Bee Diseases

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Introduction

Nosemosis is a disease of adult bees. It is caused by a single-celled microsporidian parasite of the genus *Nosema*. Two species of *Nosema* are responsible for bee disorders: *Nosema apis* Zander and *Nosema ceranae.*

N. apis is a parasite of the European honey bee, *Apis mellifera*, while *N. ceranae* is a parasite of the Asian honey bee, *Apis cerana*, and of *A. mellifera*. *N. apis* is ubiquitous. *Nosema ceranae* has been detected in different geographically distinct populations of *Apis mellifera* in Europe, North and South America, and Asia.

The spores of *N. ceranae* are in average smaller than the spores de *N. apis* (Fries, 2013):

- *N. ceranae*: around 4.7 x 2.7 µm. For Chen (2009), their sizes vary from 3.9–5.3 µm of long to 2.0 - 2.5 µm of width.
- *N. apis*: around 6 x 3 µm.

Nosemosis disease causes colony weakening and winter or spring depopulation of varying severity, sometimes associated with non-specific clinical signs: dead bees, sluggish bees crawling on the ground, traces of diarrhoea. Diagnosis of the disease may be established based on the clinical signs observed, as the bees can withstand high infection rates without disorders being apparent.

The pathogenic effects of *N. ceranae* on colonies of *Apis mellifera* are not fully known. *N. ceranae* may be involved in bee colony weakening phenomena, when other stress factors are present.

This method describes a technique for diagnosing nosemosis by microscopic examination. It is adapted from the method referenced in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Version 2008) of the World Organisation for Animal Health (OIE). It is used to detect the presence of *Nosema* spp. and to assess the average rate of infection in bees by this agent.

The species of *Nosema* is identified by molecular biology (PCR / EURL method: ANA-I1.MOA.1100).

Warnings and safety precautions

Users of this method should be fully familiar with common laboratory practices. It is the user's responsibility to establish appropriate practices in terms of health and safety, and to ensure compliance with the national regulations in force.

It is essential that the manipulations carried out in accordance with this method are performed by personnel who have received appropriate training.

Handling and disposal of materials likely to be contaminated: the laboratory must implement measures that take these risks into account to ensure that *Nosema* spp. is not disseminated in the environment.

1 Purpose and scope

The method described is a technique for diagnosing *N. apis* nosemosis in a colony with clinical signs of this disease.

The procedure developed below is a diagnostic technique adapted from the method referenced by the World Organisation for Animal Health (OIE), 2008 (Chapter 2.2.4). It enables the average rate of infection of bees by *Nosema* spp. to be assessed. The species of *Nosema* is identified by molecular biology (PCR).

2 Reference documents

[1] World Organisation for Animal Health (OIE), 2008. Nosemosis of honey bees. Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.2.4.

[2] EURL for Bee Health, protocol instruction: ANA-I1.MOA.1100. *Nosema* species identification using PCR (in-house method).

3 Terms, acronyms and definitions

OIE: World Organisation for Animal Health

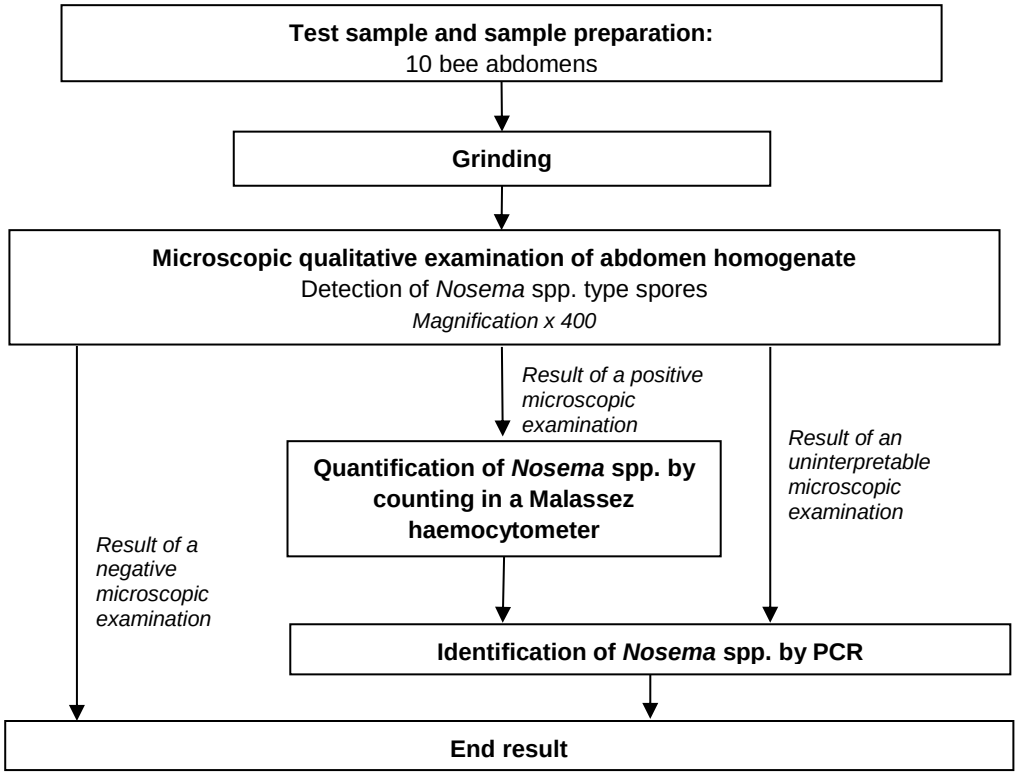
EURL: European Union Reference Laboratory

PCR: Polymerase chain reaction

4 Principle of the method

The purpose of the analysis is to assess the average rate of infection of adult bees by *Nosema* spp. This is determined from a sample of abdomen homogenate. The assessment is either qualitative, or quantitative. In the latter case, the counting takes place using a Malassez haemocytometer.

Figure 1 - Flowsheet of the method



5 Reagents

<u>Caution</u>: Trade names or commercial suppliers may be mentioned in the description of the products needed for the implementation of this method. This information is provided for the users of the method and does not in any way imply that ANSES recommends the exclusive use of these products. Equivalent products may be used if it is demonstrated that they yield the same results.

- **Water**

Use ultrapure water (distilled or demineralised water or water of equivalent purity).

6 Equipment and materials

<u>Caution</u>: Trade names or commercial suppliers may be mentioned in the description of the equipment and materials needed for the implementation of this method. This information is provided for the users of the method and does not in any way imply that ANSES recommends the exclusive use of these materials. Equivalent materials may be used if it is demonstrated that they yield the same results.

- Tweezers
- Straight dissection scissors

- Mortar and pestle (100 ml)
- Single-use pipette (5 ml and 10 ml)
- Microscopic slides and coverslips
- Malassez haemocytometer for counting
- Inoculation loop (10 µl)
- Pasteur pipette
- Centrifuge tube (50 ml)
- Cotton filtration fabric
- Vortex
- Optical microscope (x 400)
- Pulse meter
- Centrifuge
- Laboratory gloves

7 Samples

7.1 Conditions for acceptance of samples

It is important that the Laboratory receives a sample representative of the observed disorders that has not been damaged or modified during transport or storage.

The sample must contain at least ten whole bees.

When received at the laboratory, samples must not be in a state of decomposition (presence of mould in particular) and must be in sufficient quantity and able to be used for the analysis.

Indeed, the presence of microscopic fungi (other than *Nosema*) or yeasts can make the identification and counting of spores of *Nosema* spp. difficult.

7.2 Storage of samples prior to analysis

Prior to analysis, the samples must be stored under the following conditions:

- Refrigerated at around +4°C if the analysis is conducted on the day following receipt of the samples;
- Frozen at around -20°C if the analysis is deferred.

7.3 Storage of samples after analysis

The samples must be stored frozen at around -20°C.

8 Procedure

8.1 Identification of the presence of *Nosema* spp.: qualitative examination

- Take 10 bees and place them in a container such as a Petri dish.

- Using the scissors and the flexible tweezers, remove the abdomens by cutting at the petiole.
- Place the abdomens in the mortar, use the pipette to add 5 ml of ultrapure water.
- Grind with the pestle (crush the abdomens thoroughly).
- Using an inoculation loop, place 10 µl of the suspension on a microscope slide, cover with a coverslip.
- Examine under the microscope with 400x magnification.
- In the case of a positive result, proceed to the quantitative examination for the counting of spores.

8.2 Counting of *Nosema* spp. spores: quantitative analysis

- **Filtration of the abdomen homogenate**

Filter the suspension in the centrifuge tube using the folded fabric filter (two thicknesses of compress).
Rinse the mortar and pestle with 5 ml of ultrapure water and add the rinse water to the filtrate.
Press the filter with the pestle used for grinding, in order to fully extract the suspension.

- **Centrifugation**

Centrifuge for 6 minutes at 800g.

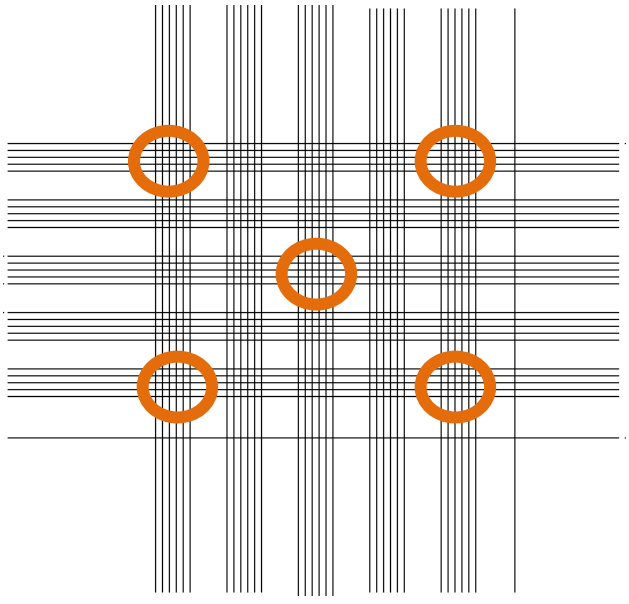
- **Resuspension of the pellet**

Remove the supernatant by effusion.
Resuspend the pellet in a homogeneous suspension with 10 ml of ultrapure water using the vortex.

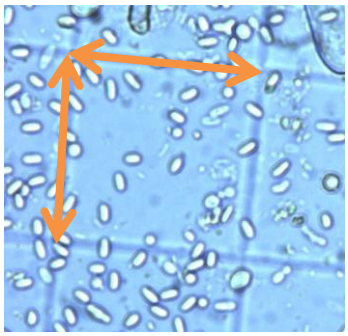
- **Counting using the Malassez haemocytometer**

Use a wetted fingertip to lightly dampen the two dishes framing the gridded area of a Malassez haemocytometer.
Carefully cover with the coverslip and push down.
Leave to dry for around 30 seconds.
Use a micropipette or Pasteur pipette to collect between 20 and 30 µl of solution. Allow it to diffuse under the coverslip by capillary action in order to fill the space between the 2 channels, avoiding any overflow into the channels. Leave to rest for a few minutes (about 4 minutes) and observe.

Use the pulse meter to count the number of spores in 5 rectangles (made up of 20 squares). The rectangles to be counted are always chosen in the same way with respect to the grid lines, like the representation of the number 5 on a domino tile:



For any spores straddling the boundary lines of the squares, only those spores straddling the upper and left lines of each square are counted:



The average n of spores per rectangle is calculated.

The number of spores N per bee is:

N = [n] x 10^5 spores/bee

Notes:
- It is possible to carry out two counts on Malassez cells, from two sets of sampling taken from the abdomen homogenate. In this case, the result of the analysis is the average of these two counts.
- In the case where spores are detected but not quantifiable, *i.e.* in the presence of rare spores outside the counting areas of the cell, the analytical result to be indicated is: <2E+04 spores per bee (the 2E+04 load corresponding to the detection of a spore on the counting area).

8.3 Description of the pathogen

The spore is oval-shaped. It is about 5–7 µm long and 3–4 µm wide (*Nosema ceranae* is slightly smaller than *Nosema apis,* but species differentiation is difficult using light microscopy, especially as mixed infections occur). It is optically transparent and refractive (Figure 2).

The spores of *Nosema* must be differentiated from other microscopic fungi (present, for example, in the case of moulds), yeasts or other particles, which may also be present in the analysed bees.

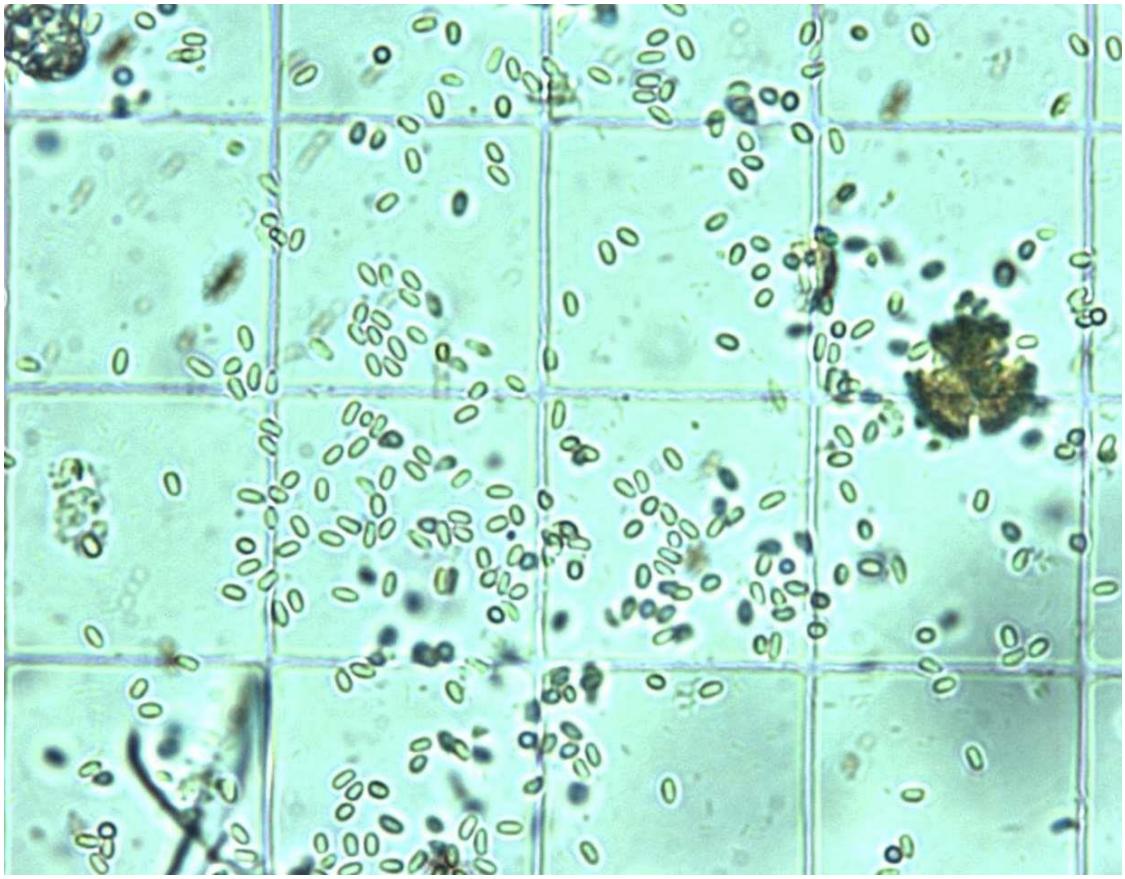


Figure 2 - *Nosema* spp. spores under the microscope on a Malassez haemocytometer (x 400)

9 Results

9.1 Quality control

In the case of an uninterpretable result (*i.e.* uncertainty about the morphological identification and therefore the actual presence of *Nosema* spores), identification of *Nosema* may be confirmed by PCR.

9.2 Calculations and reporting of results

- **Qualitative diagnosis**

The result of the qualitative diagnosis is presented as follows:

Result of the microscopic examination	Expression of the analytical result in the report Analysis parameter = *Nosema* spp. spores
Identification of *Nosema* spp. spores	Detected
Absence of *Nosema spp* spores	Not detected
Difficulty to interpret (presence of elements likely to be confused with *Nosema* spp. spores)	Inconclusive

In the case of inconclusive result (*i.e.* uncertainty about morphological identification and therefore about the very presence of *Nosema* spores), the identification of *Nosema* can be confirmed by PCR (cf. EURL protocol ANA-I1.MOA.1100).

- **Quantitative diagnosis**

The result of the quantitative diagnosis is presented as follows:

Result of the quantitative examination	Expression of the analytical result in the report Analysis parameter = number of *Nosema* spp. spores per bee
Count carried out (*i.e.* positive qualitative result): indicate the number N of spores per bee	N (or 2E+04 spores per bee if only rare spores are detected outside the counting area)
Absence of *Nosema* spp. spores	Not applicable
Inconclusive result	Not applicable

In the case of a positive result, discrimination between *N. apis* and *N. ceranae* may be performed by molecular biology using PCR.

- **In case of inconclusive results**

In case of inconclusive results (presence of elements likely to be confused with *Nosema*), the following procedure will be implemented:

1) If the sample quantity is sufficient: repeat the analysis with 10 new bees.

2) If the result remains uninterpretable or if a new analysis is not possible, perform a count of the number of microscopic elements likely to be confused with *Nosema*. If the assessed load is:

- greater than 2 x 10^4 / bee (which approximately corresponds to the detection limit of EURL PCR method ANA-I1.MOA.1100): molecular analysis can be performed to confirm the identification of *Nosema*;

- less than 2 x 10^4 / bee: the number of «uninterpretable» microscopic elements being lower than the detection limit of the EURL PCR method, molecular analysis will not confirm the identification of *Nosema*. This load being low, it can be considered as not significant from a diagnostic point of view and not explanatory of the disorders observed on the apiary (cf. next paragraph).

9.3 Guidelines for expressing opinions and interpretations

Interpretations, taking into consideration the results of the different analysis performed (microscopy and/or molecular diagnosis / ANA-I1.MOA.1100 protocol), are expressed according to the decisions rules described as follows:

		PCR result					
		Analysis not performed	***Not detected***	***N. apis detected***	***N. ceranae detected***	***N. apis et N. ceranae detected***	**Inhibited PCR**
Result of the microscopic examination	**Not detected**	(1)					
	Detected < 2 x 10^4 sp./bee	(2)					
	Detected > 2 x 10^4 sp./bee	(2)	(1) (3)	(4)	(5)	(6)	(7)
	Uninterpretable	(8)	(1) (3)	(4)	(5)	(6)	(9)
	Analysis not performed		(1)	(4)	(5)	(6)	(9)

Note: The load of 2 x 10^4 sp./bee is the PCR detection limit.

(1): “Negative screening for nosemosis.”
(2): “Infection by *Nosema* spp.”
(3): “*Nosema apis* and *Nosema ceranae* not identified.”
(4): “Infection by *Nosema apis*.”
(5): “Infection by *Nosema ceranae.”*
(6): “Infection by *Nosema apis* and *Nosema ceranae*.”
(7): “Infection by *Nosema* spp. *Nosema* species identification not possible (inhibited PCR reaction).”
(8): “Inconclusive screening for nosemosis.”
(9): “Inconclusive screening for nosemosis (inhibited PCR reaction).”

Depending on the information and clinical signs mentioned in the case history, opinions may be expressed based on the rate of infection of bees and the results of *N. apis and N. ceranae* typing by PCR.

The clinical signs associated with *Nosema apis* nosemosis are as follows (Fernandez et al., 2007):

- Dead bees in front of and/or around the hive,
- Sluggish bees,
- Depopulation (not enough bees to cover the brood),
- Bees hanging onto blades of grass,
- Traces of diarrhoea in front of and/or on the walls of the hive,

- Bees forming a circle.

In the case of clinically declared *N. apis* nosemosis, the rate of infection is generally higher than 1 million *N. apis* spores per bee.

As the clinical signs of nosemosis are non-specific, a differential diagnosis with other bee diseases could be performed depending on the case history (*e.g.* screening for tracheal acariosis, chronic paralysis, toxicological analyses).

The pathogenicity of *N. ceranae* is the subject of much debate within the scientific community. In the current state of knowledge, *N. ceranae* may be involved in weakening and depopulation phenomena, especially when other stress factors are present. The results of epidemiological investigations showed a high prevalence of *N. ceranae* infection in French apiaries, without apparent clinical signs and with rates of infection that are sometimes high (Hendrikx, 2015).

10 Performance characteristics of the method

Method validated by use.

References

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