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Ministry of Environment Water & Agriculture

Standard operating procedures for assessment of nosema disease: identification and quantification by microscopic examination BEE/051/2022/2

Strengthening MoEWA's Capacity to implement its Sustainable Rural Agricultural Development Programme (2019-2025) (UTF/SAU/051/SAU)

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1. Introduction

Nosemosis is worldwide adult honeybee disease caused by two different species of fungus *Nosema apis* and *Nosema ceranae*. The diseases propagate by spores and infection of the bees mainly occurs by the oral ingestion of spore-contaminated honey, pollen or water, faeces and by means of trophallaxis. The spores can remain infectious for up to 5 years. Nosemosis can be aggravated by different stresses (e.g. phytoterapeutic products (pesticides), scarcity of honey and pollen, seasonal weather conditions, and the presence of other diseases (varroa, viruses and amoeba). Nosemosis is contributing for the death of large number of colonies annually worldwide and its implicated as one the factor for colony collapse disorder. The current widely occurring nosemosis (in the KSA) is mostly due to *Nosema ceranae* which is affecting the bee colony without showing noticeable clinical signs/symptoms. So, it is important to conduct diagnosis of bee samples to early detect the disease and to take the necessary prevention and control measures. Diagnosis of nosemosis can be done by optical microscopy. However, to identify whether the Nosema species in the outbreak is *N. apis* or *N. ceranae*), requires adopting of molecular PCR techniques.

This Standard Operational Procedures (SOP) is prepared to guide experts involved in diagnostic survey on the identification and quantification of Nosema prevalence in the Kingdom of Saudi Arabia by optical microscopy. The SOP is also useful to guarantee and monitor the quality of the diagnostic survey. The SOP is prepared through referring recent literatures and adopting of the latest publication of (ANSES, 2020; Human et al., 2013; OIE Manual, 2021).

Along with the SOP detail checklist is also provided to assist the field experts to collect the necessary information about the sampled apiaries and sampled colonies status. This information is useful to understand the extent of the impact of the prevalence of the disease and to know well the associated factors contributing for the occurrence and dissemination of the disease.

2. Clinical signs and symptoms of Nosemosis

Some of the major clinical signs (symptoms) of colonies infected by *Nosema* spp. depend on the species of Nosema that is involved:

Nosema apis

- Trembling (shaking) of honeybee workers,
- Swollen (enlarged) abdomen,
- Some bees can be observed unable to fly or paralysis,
- Dead bees around the hive can be obtained,
- Presence of faecal marks on the combs and in front of hives,

- Declining in brood production,
- Gradual dwindling of bee colonies,
- Slow colony growth (particularly in spring).

Nosema ceranae

Colonies infected with *N. ceranae* do not show clear and immediate symptoms or clinical signs. However, the infected colonies may exhibit the following characters:

- Gradual declining of adult bees (depopulation) in a colony,
- High number of autumn and winter colony mortality (at apiary level),
- No faecal marks,
- The colony gradually die without showing any clinical signs or symptoms.

Differentiation between the Nosema species

The differentiation of the Nosema species can be done through measuring the sizes of the spores under light microscope using ocular micrometer. The spores of *N. ceranae* are in average smaller than the spores de *N. apis*. The size of *N. ceranae*: around 4.7 x 2.7 µm. which varies from 3.9–5.3 µm of long to 2.0 - 2.5 µm of width, while, *N. apis is* around 6 x 3 µm (Chen, 2009). But the accurate differentiation between the two species can be done only through genetic analysis, Polymerase Chain Reaction (PCR) tests.

3. Equipment and reagents required for identification and quantification of Nosema species

a) Reagent

Water: Ultra-pure or distilled water

b) Equipment and materials

- Forceps
- Straight dissection scissors
- Mortar and pestle (100 ml),
- Single-use pipette (5 ml and 10 ml)
- Microscopic slides and coverslips
- Malassez haemocytometer for counting of spores
- Inoculation loop (10 µl)
- Pasteur pipette
- Centrifuge tube (50 ml)
- Cotton filtration fabric
- Vortex

- Optical microscope (x 400)
- Pulse meter
- Centrifuge
- Test tubes for bee sample collection
- Laboratory gloves.

4. Sampling procedures and precautions

4.1 Sampling procedures

The degree of Nosema spp. infection in a colony can be described through the average number of spores per bee. According to Analytical Method for Animal Health, (ANSES, 2020) the possibilities of using 10 bees per colony has been suggested, while according to the OIE (2021), However, it is suggested to use up 60 bees per colony to obtain a more accurate result.

In one apiary, samples should be taken at least from 5 different randomly selected colonies that are located at different directions of the apiary to represent all colonies in apiary. However, the number of samples can be adjusted depending on the number of colonies in apiary and also the available time and resources to conduct the diagnosis. For each sampled colony reference (code) number should be assigned and labeled on the sample container and the corresponding number should be correctly recorded on the form or on excel sheet for further references.

From each colony at least 60 old bees (foragers) should be collected and the remain bees from analysis should be kept in the refrigerator for further references or in case required to repeat the test. The bee samples collected from one colony should be diagnosed and reported separately. Besides sampling, it is useful to take note or recordings if any clinical signs and symptoms of the sampled colonies and other possible associated factors (see the checklist in the excel sheet).

4.2 Necessary precautions during bee sample collection

- During collection of bees for sampling, only the oldest and forager honeybees should be targeted.
- Forager bees should be sampled outside the hive entrance, or from peripheral frames if weather does not permit flight conditions.
- Sampling should be done during the bee's flight and foraging time,
- Young bees performing orientation flight around the hive should be avoided, such bees can be recognized by the hovering behavior in large numbers outside the entrance. In such conditions sampling should be avoided.
- During non-flight conditions older bees can be collected at the peripheral combs away from brood combs to avoid sampling of newly hatched bees.

5. Storage of samples prior to analysis and after analysis

If the samples are going to be analyzed immediately, it can be kept in refrigerator at around +4°C

If it is not possible to analyze the samples immediately it should be kept frozen at around -20°C

After analysis the remain samples should be stored at around -20°C.

6. Procedure for identification (qualitative and quantitative examination)

6.1 Identification of the presence of Nosema spp. (qualitative examination)

- Take at least 10 bees (60 bees for the more accurate result is also recommended) and place them in a Petri dish or any clean container,
- Remove the abdomens of 10 bees by cutting at the petiole using scissors and flexible forceps,
- Place the abdomens in the mortar then, add 5 ml of ultra-pure water using pipette,
- 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 the sample under the microscope with 400x magnification.
- If the sample is a positive result, proceed to the counting of spores to determine the quantity of spores per bee.

6.2 Quantitative analysis: counting of Nosema spp. spores

6.2.1 Filtration of the abdomen homogenate

- Filter the grinded abdomen (suspension) in the centrifuge tube using the folded fabric filter.
- Rinse the mortar and pestle with 5 ml of ultra-pure 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.

6.2.2 Centrifugation

Centrifuge the filtrate for 6 minutes at 800g (relative centrifuge force).

6.2.3 Resuspension of the pellet

- After centrifuge remove the supernatant by effusion.
- Resuspend the pellet (the sediment) by adding 10 ml of ultra-pure water,
- Then homogeneous the suspension using vortex.

6.2.4 Counting of spores using the Malassez haemocytometer

- Carefully clean haemocytometer and cover glass with lens paper with sterilized distilled water to avoid contamination or counting errors.
- Then dry with lens paper.
- Use a wetted fingertip to slightly moisten the edges the two dishes framing the gridded area of a Malassez haemocytometer

- Then carefully cover the haemocytometer gridded area with the coverslip and push down. Make sure to use the provided cover glasses - these glasses are thicker than the standard cover glasses so that surface tension will not deform them.
- Press firmly until the slide and cover come into contact properly.
- Leave to dry for around 30 seconds.
- Use a micropipette or Pasteur pipette to collect between 20 and 30 µl of suspension solution and carefully allow the solution 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 about 4 minutes.
- Use the pulse meter to count the number of spores in different 5 rectangles (made up of 20 squares) (Fig.1).
- The rectangles to be counted are always chosen in the same way with respect to the grid lines.

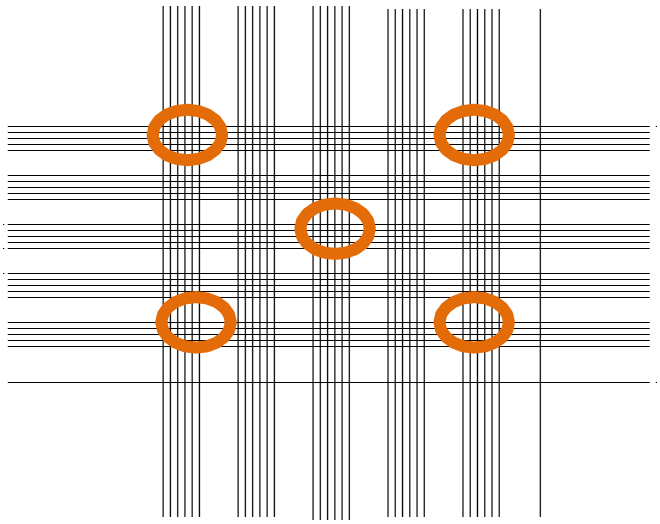

Fig. 1 Malassez haemocytometer grid

For any spores straddling on the boundary lines of the squares, only those spores straddling on the upper and left lines of each square are counted and the spores stranded at right and bottom the boundary lines are ignored Fig. 2.

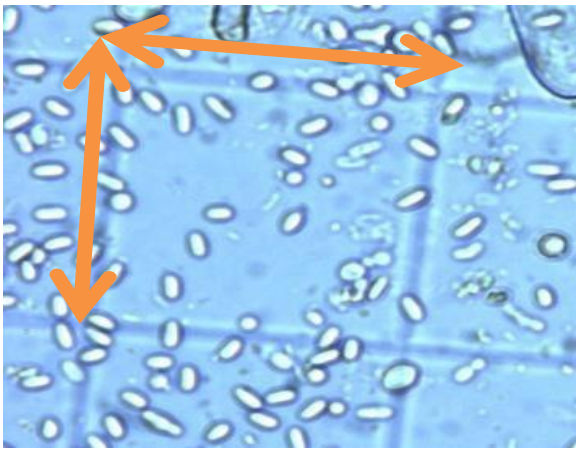

Fig 2 Shows spores stranded on the boundary lines and the upper and left are counted while spores stranded on the right and bottom lines are not considered.

The average **n** of spores per rectangle is calculated by adding all spores counted from 5 rectangles then divided by 5

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 same 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).

7. References

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