Journal of Microbiological Methods 184 (2021) 106183

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

An international inter-laboratory study on *Nosema* spp. spore detection and quantification through microscopic examination of crushed honey bee abdomens

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ARTICLE INFO

Keywords:
Diagnosis
Interlaboratory comparison
Microscopic counting
Microsporidia
Nosema spp.

ABSTRACT

Nosemosis is a microsporidian disease causing mortality and weakening of honey bee colonies, especially in the event of co-exposure to other sources of stress. As a result, the disease is regulated in some countries. Reliable and harmonised diagnosis is crucial to ensure the quality of surveillance and research results. For this reason, the first European Interlaboratory Comparison (ILC) was organised in 2017 in order to assess both the methods and the results obtained by National Reference Laboratories (NRLs) in counting *Nosema* spp. spores by microscopy. Implementing their own routine conditions of analysis, the 23 participants were asked to perform an assay on a

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https://doi.org/10.1016/j.mimet.2021.106183
Received 13 January 2021; Received in revised form 24 February 2021; Accepted 24 February 2021
Available online 27 February 2021

panel of ten positive and negative samples of crushed honey bee abdomens. They were asked to report results from a qualitative and quantitative standpoint. The assessment covered specificity, sensitivity, trueness and precision. Quantitative results were analysed in compliance with international standards NF ISO 13528 (2015) and NF ISO 5725-2 (1994). Three results showed a lack of precision and five a lack of trueness. However, overall results indicated a global specificity of 98% and a global sensitivity of 100%, thus demonstrating the advanced performance of the microscopic methods applied to *Nosema* spores by the NRLs. Therefore, the study concluded that using microscopy to detect and quantify spores of *Nosema* spp. was reliable and valid.

1. Introduction

Nosemosis is a global disease of adult honey bees. It is caused by a spore-forming unicellular parasite of the Microsporidia group *Nosema*. However, a recent study based on a molecular comparison of the SSU rRNA gene proposed a new definition of the *Nosema* clade (Tokarev et al. 2020). The two main species of *Nosema* causing disorders in honey bees worldwide are *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Fries et al. 1996). Another species, *Nosema neumanni*, has been found in honey bees in Uganda (Chemurot et al. 2017) but the implications of infections with *N. neumanni* still have to be studied. *Nosema apis* and *N. ceranae* multiply in the epithelial cells of the posterior ventricle region. The spores present in the lumen of the digestive tract germinate and release polar filaments that mechanically perforate epithelial cells and through which the sporoplasm enters the cell to multiply. The cell is damaged as a large number of spores is generated. Two types of spores are produced: the primary spores that are capable of transmitting infection to adjacent cells, and the mature environmental spores that may be voided with the faeces or stay in the gut to start a new multiplication cycle (Fries 1988; Goblirsch 2018; Higes et al. 2007). *Nosema apis* is a parasite of the European honey bee (*Apis mellifera*) while *N. ceranae,* originally described in the Asian honey bee (*Apis cerana*) but also detected in *A. mellifera* populations in a number of geographically distant regions: Europe (Higes et al. 2006), South and North America (Calderón et al. 2008; Chen et al. 2008), Africa and Asia (Chen et al. 2009). *Nosema* species (spp.) spores have an ovoid morphology. *Nosema ceranae* spores measure approximately 4.7 × 2.7 μm, making them smaller on average than *N. apis* spores, which measure approximately 6 × 3 μm (Fries et al. 2013; Fries et al. 1996; Zander and Böttcher 1984). However, this slight difference in size is not sufficient for a differential diagnosis in routine microscopy analyses. Molecular methods (PCR) are therefore required to identify *Nosema* spp.

Nosema infection between adult bees is spread by the exchange of spores during feeding (trophallaxis) or comb-cleaning. Contaminated beekeeping equipment, honey stores and water also play a role in the transmission of the disease. *Nosema apis* spores expelled with faeces remain viable for over a year. They also remain contagious in honey (MacInnis et al. 2020) and in bee bodies.

The clinical signs of nosemosis are not specific. High infection rates can weaken the colony, leading to varying levels of depopulation in winter or spring. In the case of *N. apis*, dead bees, bees crawling on the ground and traces of diarrhoea may be observed on or around the hive. In contrast, the pathogenic effects of *N. ceranae* on *A. mellifera* colonies are not clearly understood. *N. ceranae* is thought to contribute to colony weakening, particularly in the presence of other sources of stress (Alaux et al. 2010; Doublet et al. 2015; Vidau et al. 2011; Zheng et al. 2015). Climate is also thought to have an effect on the pathogenicity of *N. ceranae*. While it is assumed that in warm areas the chronic stress caused by *N. ceranae* infections might favour colony death (Higes et al. 2008; Martín-Hernández et al. 2018), it was shown on the other hand that *N. ceranae* may be more virulent and better adapted than *N. apis* in cold climates (Emsen et al. 2016). It should also be noted that bees can sustain high infection rates of *N. apis/N. ceranae* without apparent symptoms (Meana et al. 2010) (unpublished data). Given the difficulties of diagnosis, laboratories need to detect and quantify *Nosema* spp. spore loads in honey bees and to establish a differential diagnosis with other adult honey bee diseases causing similar disorders (*e.g.* tracheal acariasis, amoebiasis, chronic paralysis, intoxication, *etc.*).

Nosemosis is not covered by European Union regulations, nor is it included in the list drawn up by the World Organisation for Animal Health (OIE). However, the disease is regulated at national level in some countries. The OIE Manual of Diagnostic tests and Vaccines for Terrestrial Animals describes a diagnosis method for *Nosema disease* based on the detection and the quantification of spores by microscopy (World Organisation for Animal Health (OIE) 2018). A number of official laboratories have implemented this method.

In response to diagnostic and health issues, and to ensure the quality of the analytical results obtained within the European Union (EU), the European Union Reference Laboratory (EURL) for Bee Health, located in the laboratory of the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) in Sophia-Antipolis (France), organised a InterLaboratory Comparison (ILC) using microscopy to detect and count *Nosema* spp. spores in crushed bee samples. This was the first test of this method organised by the EU. All the EU National Reference Laboratories (NRLs) were invited to participate in the ILC. The overall objective was to assess the ability of laboratories to establish a correct result using their routine analysis. Four criteria were evaluated: sensitivity, specificity, trueness and precision of the results. At the same time, a survey was conducted within the network of EU NRLs in order to collect information on their analytical methods with the perspective of a possible harmonisation.

2. Materials and methods

2.1. Participating laboratories

In June 2017, the EURL for Bee Health organised an ILC. A total of 23 NRLs for Bee Health took part in this trial, all from EU member states. In order to ensure the confidentiality of results, each participating laboratory was assigned an individual random code number.

2.2. Reference methods

Two reference methods were used to characterise and check the homogeneity and stability of the samples used in the ILC: i) a microscopy-based method to detect and count *Nosema* spp. spores, and ii) a PCR-based method to confirm detection of *Nosema* spp. (this method was only used to characterise the samples, *i.e.* to verify their negative or positive status). The EURL is accredited by the French Accreditation Committee (COFRAC) for these two methods in compliance with the international standard ISO/IEC 17025 on “General requirements for the competence of testing and calibration laboratories” (NF EN ISO/IEC 17025 2005).

The microscopic method is based on the procedure developed by Cantwell (Cantwell 1970) and recommanded by the OIE in the Terrestrial Manual (World Organisation for Animal Health (OIE) 2018) intending to detect and evaluate the average infection rate of bees by *Nosema* spp. spores using microscopy. In brief, the procedure involves crushing bee abdomens (60 bees) with a mortar and a pestle in ultrapure water at a rate of one millilitre (1 mL) per bee. The suspension is filtered through two layers of muslin (thin loosely woven cotton fabric) and centrifuged for six minutes at 800 ×*g* to eliminate large debris and to

Table 1
Composition of the test panel of samples sent to participating laboratories.

Samples	Microscopic examination			*Nosema* species	Criterion evaluated
	Detection	Counting[b] (spores/ml)	SD[c] (spores/ml)		
POS1	Positive	3.47E+06	2.83E+05	*N. ceranae*	Sensitivity
POS2[a]	Positive	4.73E+05	8.39E+04	*N. ceranae*	Sensitivity
POS3[a]	Positive	1.84E+06	2.01E+05	*N. ceranae*	Sensitivity
NEG1	Negative	–	–	–	Specificity
NEG2	Negative	–	–	–	Specificity
NEG3	Negative	–	–	–	Specificity
Lure	Positive or negative	7.04E+05 –	1.96E+05 –	*N. ceranae* –	not evaluated

[a] Samples tested in triplicate.
[b] Mean of the homogeneity study results.
[c] Standard deviation evaluated within the homogeneity study.

purify the spores. The pellets are then resuspended to a homogeneous suspension in order to restore the initial dilution of 1 mL per bee. Finally, the sample is placed in a calibrated haemocytometer (Malassez counting chamber) and the microscopic examination is performed to detect and count *Nosema* spp. spores. The analytical results are both qualitative (negative *versus* positive) and quantitative (number *Nosema* spp. spores per mL, *i.e.* per bee, based on dilution). It should be noted that measurement uncertainty may be high for the visual counting method. It can vary depending on the number of *Nosema* spores and particles (*e.g.* pollen, yeast) present in the bee's digestive tract, which may interfere with the detection and identification of *Nosema* spores.

The main steps in the molecular method are as follows. First, 80 µL of the suspension of crushed bee abdomens, prepared for the microscopic examination, is used to extract DNA using High Pure PCR Template Preparation Kit (Roche Diagnostics). The DNA extraction is performed following the "tissue" protocol without any change. The extracted DNA is resuspended in 200 µL of elution buffer according to the manufacturer's recommendations and stored at −20 °C ± 5 °C until further analysis (used as a template in the PCR). The PCR is performed as follows: 25 µL of the reaction mixture containing 1 U Platinum Taq DNA polymerase (Invitrogen), 0.4 µm of each primer, 0.4 mM dNTPs and adjusted with nuclease-free H_2O to reach a final reaction volume of 20 µL plus 5 µL of extracted DNA. The PCR reactions were run in an Eppendorf Mastercycler® Nexus ThermoCycler under the following cycling conditions: initial denaturation at 94 °C for 2 min followed by 35 cycles of 30s at 94 °C, 30s at 62 °C and 30s at 72 °C with a final extension of 7 min at 72 °C. The PCR allows the identification of *Nosema* species (*N. apis* and *N. ceranae*) using the species-specific primers described by Martin-Hernandez (Martín-Hernández et al. 2007).

2.3. Inter-comparison samples

The samples from the ANSES collection at Sophia-Antipolis laboratory originated from diagnostic analyses, field studies, experimental infections and collaborations. The panel included two types of samples: crushed *A. mellifera* abdomens, prepared according to the reference method described in the paragraph above, and a filtered suspension of *N. ceranae* spores. The status of each batch of samples (negative or positive for *Nosema* spp., defined spore load) was based on the results obtained with the two independent methods described above. The PCR also demonstrated that all positive samples were infected by *N. ceranae*.

Three negative *Nosema* spp. samples (NEG1, NEG2 and NEG3) were prepared using bees from the ANSES experimental apiary. Three positive *Nosema* spp. samples (POS1, POS2 and POS3) with different infectious loads (3.47E+06, 4.73E+05 and 1.84E+06 spores per mL, *i.e.* per bee respectively) were included in the panel (Table 1). POS1 and POS2 were prepared using bees naturally infected with *N. ceranae* from the EURL sample collection, while POS3 was a suspension of *N. ceranae* spores, prepared from experimentally infected emerging bees and filtered through a 100 µm mesh sieve.

Each crushed sample was distributed in tubes with a volume of 400 µL per tube and the batches of tubes were stored at −20 °C ± 5 °C until shipment. The panels sent to participants included ten evaluation samples and a lure sample (Table 1). The lure sample was positive or negative, depending on the participant, was not evaluated and was added to limit the risk of interlaboratory collusion. Two positive *Nosema* spp. samples (POS2 and POS3) were sent in triplicate to assess the accuracy and the trueness of participants' results.

Table 2
Homogeneity of positive samples.

Sample code	m1	SD	SD/σpt	Criteria SD/σpt ≤ 0.3
POS1	6.54	0.02	0.11	OK
POS2	5.67	0.05	0.22	OK
POS3	6.26	0.03	0.19	OK

m1: $\log_{10}$ of the mean of the results obtained by the homogeneity study.
SD: inter-sample standard deviation.
SD/σpt ≤ 0.3: homogeneity criterion according to Annex B of international standard NF ISO 13528.

Table 3
Stability of positive samples.

	Sample code	m1	m2	m1-m2	(m1-m2)/σpt	Criteria (m1-m2)/σpt ≤ 0.3
D0	POS1	6.54	6.50	0.04	0.17	OK
	POS2	5.67	5.63	0.04	0.17	OK
	POS3	6.26	6.22	0.04	0.23	OK
D20	POS1	6.54	6.50	0.04	0.18	OK
	POS2	5.67	5.63	0.04	0.18	OK
	POS3	6.26	6.21	0.05	0.28	OK

m1: $\log_{10}$ of the mean of the results obtained by the homogeneity study.
m2: $\log_{10}$ of the mean of the results obtained by the stability study.
(m1-m2)/σpt ≤ 0.3: stability criterion according to Annex B of international standard NF ISO 13528.

2.4. Sample homogeneity and stability

The EURL conducted homogeneity and stability tests. Homogeneity tests were performed for all sample batches between February and April 2017, *i.e.* before shipment. The homogeneity of each batch was tested by means of a duplicate analysis of ten randomly selected samples stored at −20 °C. In total, 20 results per batch were obtained. The homogeneity criterion of the negative samples was defined as a number of spores per mL (or bee) not exceeding 2E+04. This corresponds to the detection limit of the microscopic counting method using the Malassez chamber. All the negative samples met this criterion, with no *Nosema* spp. spores detected in any of the selected samples. The homogeneity of the positive samples was evaluated by calculating the standard deviations (SD) between samples in compliance with the formula set out in Annex B of international standard NF ISO 13528 (NF ISO 13528, 2015). The analysis was carried out on the number of spores per mL expressed as decimal logarithm ($\log_{10}$), in order to facilitate data analysis. The homogeneity of the inter-comparison samples was validated against a target standard deviation value (σpt) of 0.2. This value was based on the results of an inter-laboratory validation test organised in 2016 within France's official laboratory network. As specified in Annex B (paragraph B.2.2) of international standard NF ISO 13528, it was necessary for inter-sample standard deviation (SD) to fall under the critical value of 0.3 σpt. SD values were calculated for the three positive samples. They ranged from 0.02 to 0.05 $\log_{10}$, while the SD/σpt ratios were below 0.3 (Table 2). The homogeneity criterion was met and the positive samples were considered homogeneous.

Stability tests were performed on positive batches. Stability was

controlled by a duplicate analysis of three randomly selected samples stored at −20 °C ± 5 °C. In total, six results were obtained for each sample (Table 3). The tests were carried out one day after the shipment of panels to the participants (D-0) and at the end of the ILC period (deadline for sending in results) (D-20). The stability of the positive samples was evaluated in compliance with the criteria set out in Annex B (paragraph B.5.1) of international standard NF ISO 13528. The results of the stability tests at D-0 and D-20 were compared to those from the homogeneity tests. The difference between the mean of the homogeneity test results (mL) and the mean of the stability test results (m2) was less than the critical value of 0.3 σpt (*i.e.* $|mL\text{-}m2| \leq 0.3\ \sigma pt$), thus validating the stability of the positive samples during the trial period (Table 3).

2.5. Study design

The ILC was organised in compliance with the quality requirements described in international standards ISO/IEC 17043 and ISO/IEC 17025 (NF EN ISO/IEC 17025 2005, NF EN ISO/IEC 17043, 2015). The samples were packed and shipped between the EURL and NRLs in compliance with UN3373 regulations (Biological Substance, Category B).

Each participating laboratory was anonymously coded with a 1- or 2-digit random number to ensure the confidentiality of results. Each of the samples to be blind-tested was coded with the attribution of a random number between 1 and 11. Participating laboratories received inter-comparison samples with a laboratory code on each tube. After receiving the package, the laboratories were required to store the samples at −20 °C ± 5 °C until analysis and to send back their results within 15 days. They were asked to report the results: i) qualitatively (detected *versus* not detected, with samples of $\leq 2 \times 10^4$ spores/mL being considered as "not detected"), and ii) quantitatively (number of *Nosema* spp. spores per mL, corresponding to the number of *Nosema* spp. spores per bee). Participating laboratories were required to conduct calculations in compliance with their own analytical methods and, more particularly, according to the type of counting chamber used for analysis.

Results were evaluated according to four performance criteria:

1) Specificity, *i.e.* the ability of the laboratory to give a negative result for a negative sample (NF EN ISO 22117 2010). The expected specificity rate was 100% of negative results.
2) Sensitivity, *i.e.* the ability of the laboratory to give a positive result for a positive sample (NF EN ISO 22117 2010). The expected sensitivity rate was 100% of positive results.
3) Trueness, which was evaluated only for positive quantitative results by calculating the z-score in compliance with international standard NF ISO 13528 (NF ISO 13528, 2015).
4) Precision, which was evaluated only for positive quantitative results, by calculating Mandel's k-value in compliance with international standard NF ISO 5725-2 (NF ISO 5725-2 1994).

2.6. Technical survey of the analytical methods employed by the ILC participants

This study was the first step in a process to evaluate the level of harmonisation across the European NRL network for the diagnosis of *Nosema* spp. by microscopy. Participating laboratories were asked to use their own routine methods to analyse the ILC panel of samples. Concurrently with the test, and in order to gather information on these methods, the EURL asked participants to complete an online survey (using Sphinx iQ2 software, version 7.4.0.0, Le Sphinx Développement), detailing each stage of their routine procedure, from grinding the sample to interpreting the results.

2.7. Statistical analysis of results

In the first instance, a qualitative analysis was conducted in order to evaluate the sensitivity and specificity of results. Conformity was assessed using the reference values obtained during the homogeneity study. Sensitivity and specificity rates were calculated using the formula below, set out in standard NF ISO 22117 (NF EN ISO 22117 2010):

- Sensitivity rate: $r_{SE} = n_{+}/E_{(n+tot)} \times 100\%$ (where n_{+} is the number of positive results found and $E_{(n+tot)}$ is the total number of expected positive samples).
- Specificity rate: $r_{SP} = n_{-}/E_{(n\text{-}tot)} \times 100\%$ (where n_{-} is the number of negative results found and $E_{(n\text{-}tot)}$ is the total number of expected negative samples).

The second step was to analyse the quantitative results from the positive samples included in triplicate in the panel (POS2 and POS3). The value assigned to each positive sample was established as the consensus value for the results of participants, in compliance with the procedure described in Appendix C of international standard NF ISO 13528 and corresponding to the robust average of participants. The individual results of each participant were then compared to this value, taking account of standard uncertainty. SD for the ILC assessment (σpt) was calculated using the results obtained by participants. The trueness of the results was evaluated by means of the z-score, which expressed the ratio between the observed deviation from the mean value and the standard deviation of the ILC (σpt). It was calculated in compliance with standard NF ISO 13528 (NF ISO 13528, 2015). The level of precision was evaluated through the graphical representation of Mandel's k-values, in compliance with international standard NF ISO 5725-2 (NF ISO 5725-2 1994). Mandel's k-values are intra-laboratory statistics calculated for each sample and each participant. They correspond to the ratio between the standard deviation of the participant's results and the average standard deviation of the sample.

Table 4
Sensitivity and specificity rates attained by each participating laboratory.

Laboratory Code	Sensitivity[a] (%)	95% confidence interval (%)	Specificity[b] (%)	95% confidence interval (%)
lab1	100	64.6 to 100	100	43.9 to 100
lab2	100	64.6 to 100	100	43.9 to 100
lab3	100	64.6 to 100	100	43.9 to 100
lab4	100	64.6 to 100	100	43.9 to 100
lab5	100	64.6 to 100	100	43.9 to 100
lab6	100	64.6 to 100	100	43.9 to 100
lab7	100	64.6 to 100	100	43.9 to 100
lab8	100	64.6 to 100	100	43.9 to 100
lab15	100	64.6 to 100	100	43.9 to 100
lab16	100	64.6 to 100	100	43.9 to 100
lab17	100	64.6 to 100	100	43.9 to 100
lab18	100	64.6 to 100	100	43.9 to 100
lab20	100	64.6 to 100	100	43.9 to 100
lab21	100	64.6 to 100	66.7	20.8 to 93.8
lab22	100	64.6 to 100	100	43.9 to 100
lab23	100	64.6 to 100	100	43.9 to 100
lab24	100	64.6 to 100	100	43.9 to 100
lab25	100	64.6 to 100	100	43.9 to 100
lab26	100	64.6 to 100	100	43.9 to 100
lab27	100	64.6 to 100	100	43.9 to 100
lab38	100	64.6 to 100	100	43.9 to 100
lab40	100	64.6 to 100	100	43.9 to 100
lab41	100	64.6 to 100	100	43.9 to 100
Overall	**100 (161/161)**	**97.7 to 100**	**98.6 (68/69)**	**92.2 to 99.7**

[a] Calculation of the sensitivity rate was based on 7 samples (see Table 1).
[b] Calculation of the specificity rate was based on 3 samples (see Table 1).

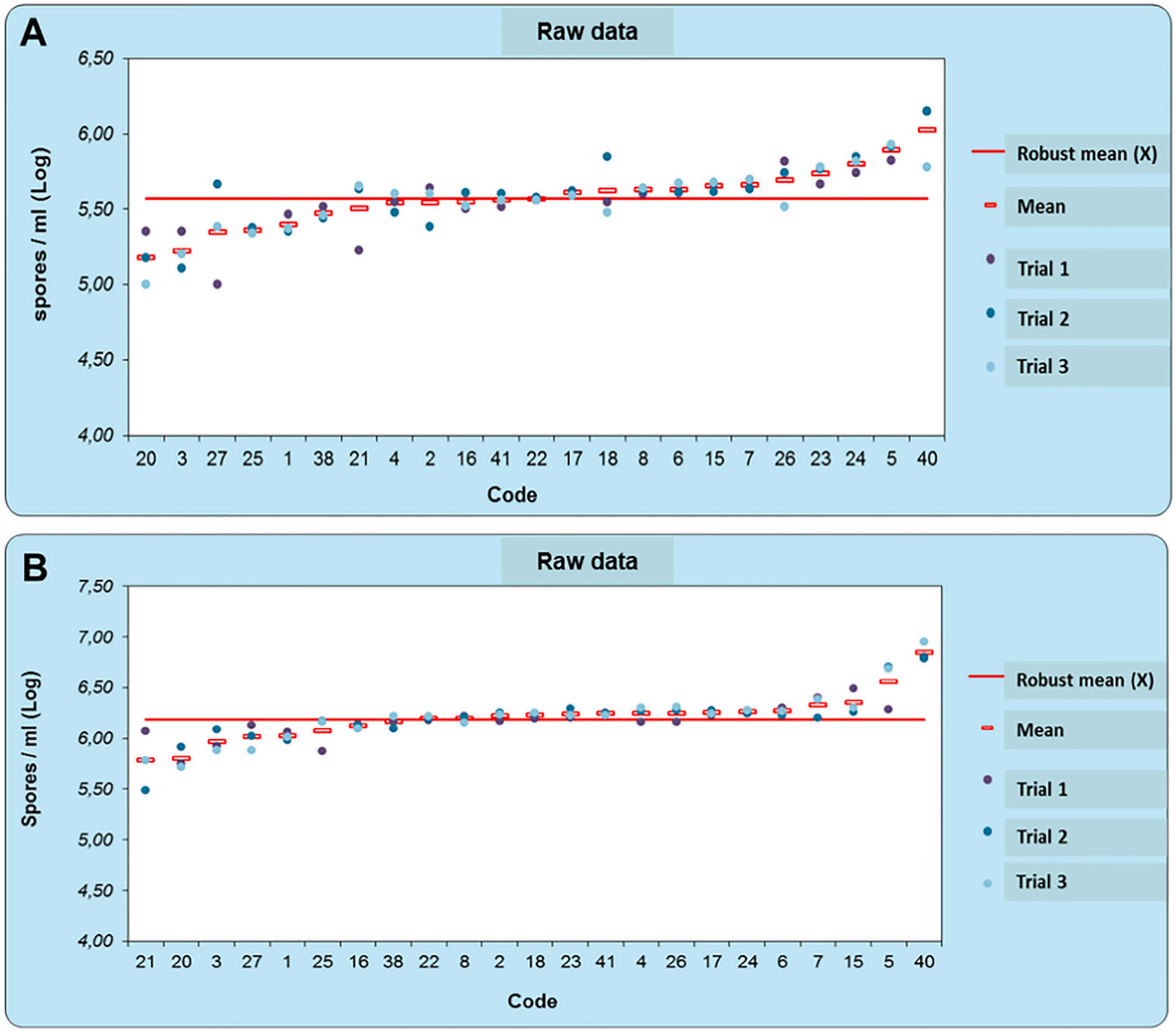

Fig. 1. Experimental results of participants when quantifying *Nosema* spp. spores in the ILC samples. Each participant tested three replicates of the POS2 sample (Fig. 1A) and three replicates of the POS3 sample (Fig. 1B). The bullet points indicate the number of *Nosema* spp. spores per bee (expressed in $\log_{10}$/bee) quantified by microscopy. The empty red box is the mean value found by each participant. The red lines indicate the robust mean (X). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Analysis of qualitative results

Participants identified all the positive samples. Sensitivity was therefore satisfactory for all the participants and complied with the expected rate of 100%. However, the analysis revealed a non-compliance in specificity for the laboratory with code No. 21, which gave a positive result for sample NEG3. Specificity was therefore satisfactory for 95.6% of participants (22 of 23).

Across the network of the 23 participating EU NRLs, the global sensitivity rate for results was 100% while the global specificity rate was 98.6% (Table 4).

3.2. Analysis of quantitative results

All the results of the participants were included in the analysis (Supplemental information, Table S1). The performance of participants was evaluated by sending three replicates of two different loaded samples (POS2 and POS3), as described in the section "Materials and methods". Fig. 1A and B show the results of the 23 participants for sample POS2 and sample POS3 respectively. The values assigned to samples POS2 and POS3 are 5.57 and 6.19 $\log_{10}$ respectively (with a standard deviation of 0.18 and 0.16 $\log_{10}$). The mean and standard deviation were estimated through a robust analysis of all participant data, in compliance with algorithm A described in Appendix C of international standard NF ISO 13528. As the uncertainties relating to the values assigned to the two positive samples could be considered as negligible, they were not included in the interpretation of the results, making it possible to use a z-score for the evaluation.

3.3. Assessment of the trueness of results

The z-score values are shown in two histograms (Fig. 2). Fig. 2A and B show the z-score of each participant for the three repetitions of results with POS2 and POS3, respectively.

As a reminder, if the z-score is 0, the measured value (x) corresponds to the assigned value (x*). The interpretation of z-scores is set out in international standard NF EN ISO/IEC 17043 as: (i) if $|z| \leq 2.0$ then the value of z is considered to be acceptable, (ii) if $2.0 < |z| < 3.0$ the value of z is considered to give a warning signal, and (iii) if $|z| \geq 3.0$ the value of z is considered to be unacceptable and generates an action signal.

Five participants delivered unacceptable results, with a $|z| \geq 3.0$: the participants with code No. 20 and No. 27 for sample POS2, the

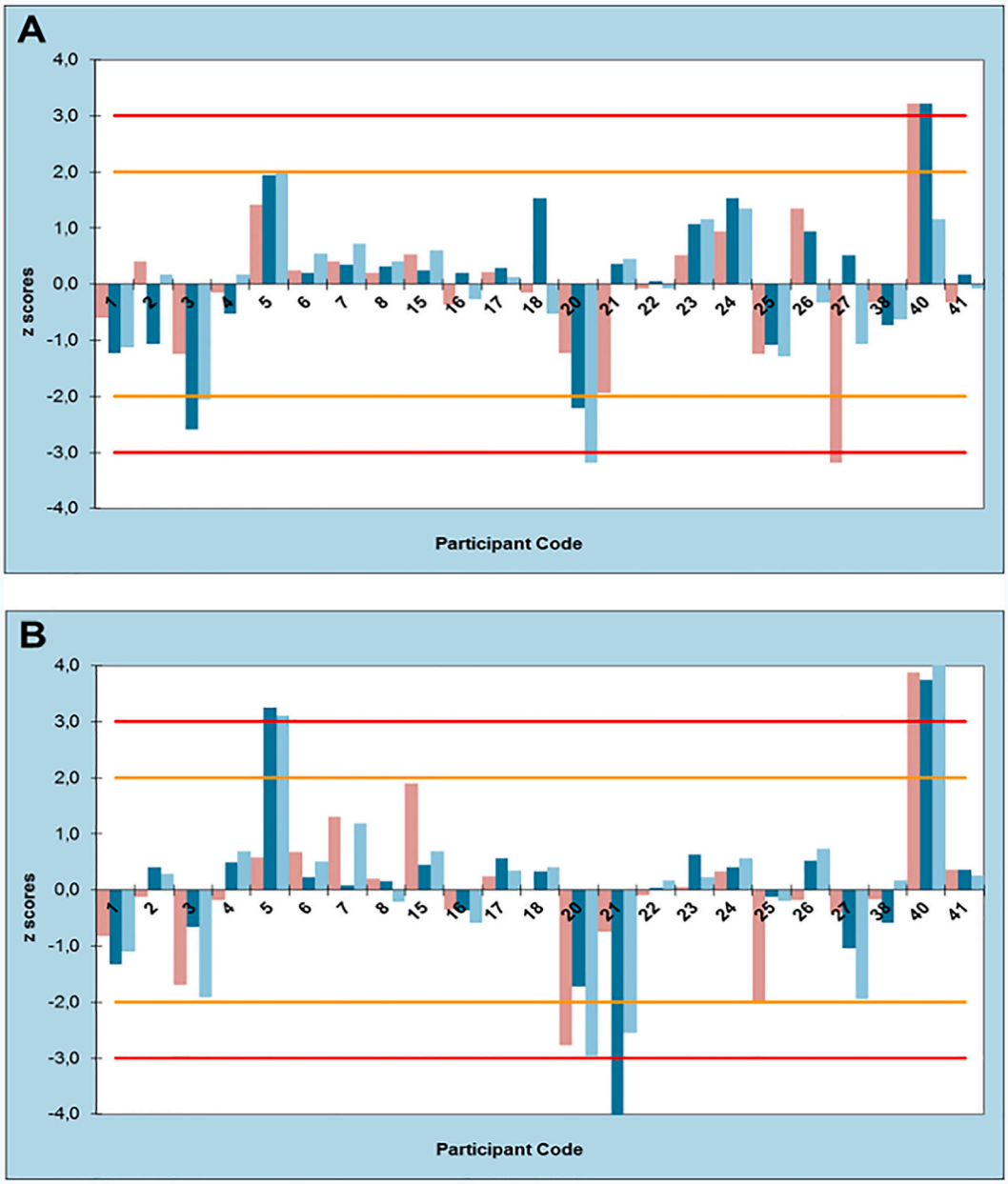

Fig. 2. *Z*-scores calculated for each participant quantifying *Nosema* spores in the inter-laboratory comparison samples. The boxes indicate the individual z-score for the three replicates of the POS2 sample (Fig. 2A) and POS3 sample (Fig. 2B) tested by each participant. The yellow and red lines indicate the limits of ±2 and ± 3 respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

participants with code No. 5 and No. 21 for sample POS3, and the participant with code No. 40 for both samples. These results were assessed as non-compliant.

At ILC level, trueness was satisfactory (*i.e.* $|z| < 3.0$) for 92.8% of the results provided by participants.

3.4. Assessment of the precision of results

For 23 participants and three repeats, the critical Mandel's k-value at the 1% significance level was 2.08. Given that less than 25% of k-values were below the critical value, the data of all participants could be used for analysis. The Mandel's k-values (Fig. 3) show that 87% of participants (20 laboratories) achieved good repeatability with both samples. Three participants encountered problems with this criterion: the participant with code No. 27 for sample POS2, and the participants with code No. 5 and No. 21 for sample POS3.

3.5. Technical survey of analytical methods employed

The online survey was sent out in June 2017. Of the 23 ILC participants, seven laboratories (30%) were accredited for the diagnosis of nosemosis by microscopy. Most methods (75%) were recommended by or adapted from the OIE Manual. The other methods were internal or based on methods published in literature. One laboratory (No. 21) was using a method for the first time with the ILC.

A wide diversity of counting chambers was used for counting *Nosema* spp. spores (Fig. 4). The Bürker and Neubauer chambers were the most frequent choices (25% and 30% respectively). It should be noted that two participants did not perform routine counting, preferring to make a semi-quantitative evaluation of the *Nosema* spp. spore load (giving rise to analytical results such as "sporadic occurrence", "weak infestation", "moderate infestation", "strong infestation", depending on the number of spores observed per microscopic field).

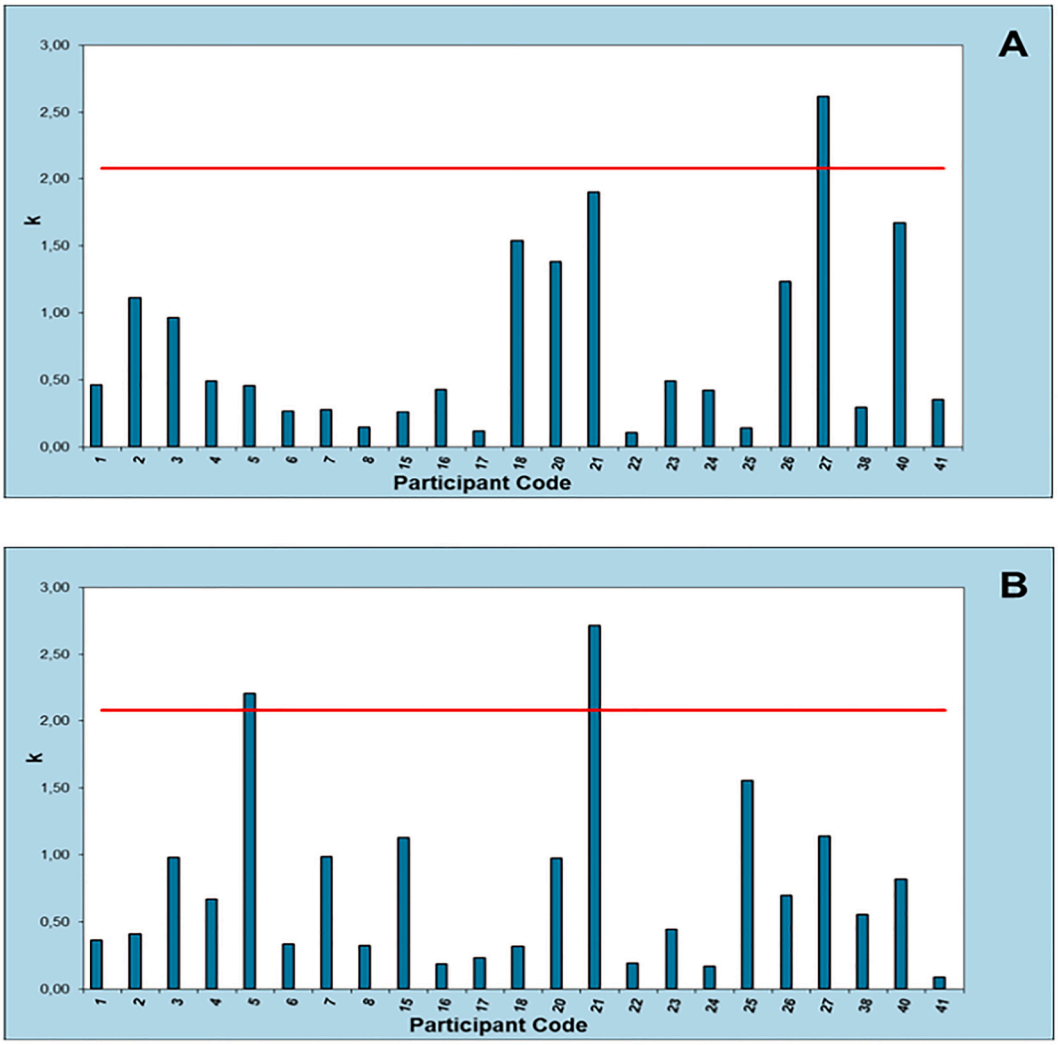

Fig. 3. Mandel's k-value calculated for each participant quantifying *Nosema* spores in the inter-laboratory comparison samples. The boxes indicate the individual z-score for the three replicates of the POS2 sample (Fig. 3A) and POS3 sample (Fig. 3B) tested by each participant. The 1% significance level is indicated by the red line (k = 2.08). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

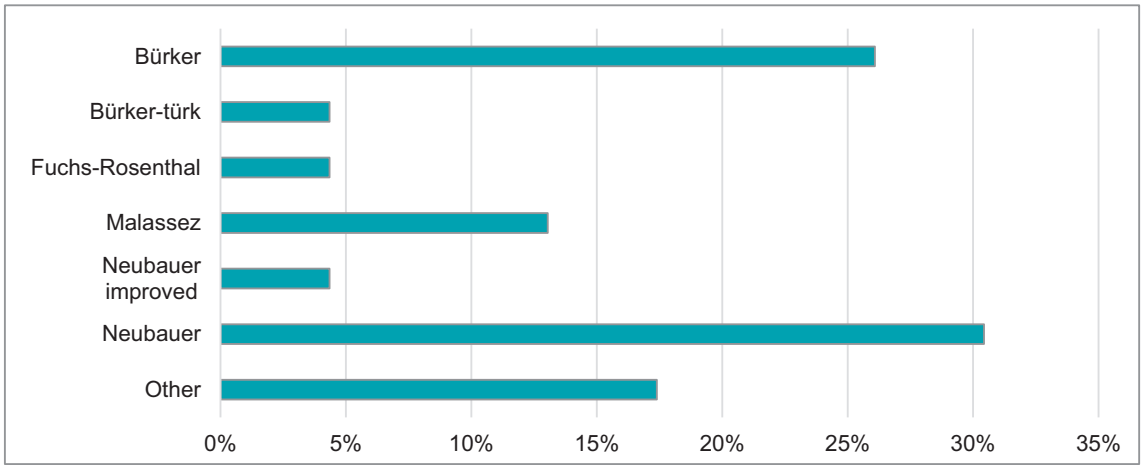

Fig. 4. Type of counting chambers used by the 23 participants.

About 60% of the laboratories used a single microscopic preparation (*i.e.* one microscopic slide prepared from the abdomen suspension), while around 40% used several slides (*i.e.* several microscopic slides prepared from the suspension) in order to calculate the mean of the different counts as their final result. Moreover, some laboratories carried out several counts on the same microscopic slide. One laboratory used

Table 5
Participant fulfilment of proficiency test criteria.

Criteria	Participant code																						
	1	2	3	4	5	6	7	8	15	16	17	18	20	21	22	23	24	25	26	27	38	40	41
Specificity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	**No**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Sensitivity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Trueness	Yes	Yes	Yes	Yes	**No**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	**No**	**No**	Yes	Yes	Yes	Yes	Yes	**No**	Yes	**No**	Yes
Precision	Yes	Yes	Yes	Yes	**No**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	**No**	Yes	Yes	Yes	Yes	Yes	**No**	Yes	Yes	Yes

The non compliant results are indicated in bold.

two preparations, each slide being counted twice by different analysts.

To finish, it should be also noted that some laboratories adjusted the spore-counting protocol depending on the sample (*e.g.* spore load). In the event of a high number of spores, for instance, 60% of laboratories said that they diluted the suspension.

4. Discussion

The ILC was carried out with 23 NRLs from the EU. Its purpose was to evaluate their level of competence in using microscopy to detect and quantify *Nosema* spp. spores in crushed honey bee abdomens. Of the 23 participants, 18 laboratories (78%) obtained compliant results for all evaluation criteria (specificity, sensitivity, trueness and precision) (Table 5). Nine results, attributed to five participants, failed to meet the defined criteria: i) one for a lack of specificity, ii) five for a lack of trueness, iii) three for a lack of precision. A lack of trueness was observed for laboratory No. 40, which globally over-estimated the number of spores in the two positive samples included in triplicate as part of the panel (POS2 and POS3). This laboratory was accredited for the method used; its procedure was based on a single count with a Malassez chamber. The results of participant No. 20 showed a lack of trueness for sample POS2, tending – in contrast – towards under-estimation. The method employed by this laboratory for the ILC relied on a single microscopic preparation counted once with a Neubauer chamber. The results of participant No. 21 showed a lack of trueness, precision and specificity. It should be noted that this laboratory did not apply a routine method, which could explain these non-compliances. Likewise, participant No. 27 encountered problems of trueness and precision. This participant did not make routine use of a counting chamber, but gave semi-quantitative results expressed by crosses in the current analysis (the number of crosses depending on the number of *Nosema* spp. spores observed in the microscopic field). A lack of trueness and precision was also observed for laboratory No. 5, although this participant was using an accredited method, based on the preparation of two microscopic slides counted twice by two different analysts using a Bürker-Türk chamber.

Following the ILC, the laboratories undertook an investigation in collaboration with the EURL to identify and resolve the causes of the non-conformities observed. In one case, the anomaly of trueness was directly linked to an error in the formula used for converting the number of spores counted with the haemocytometer in “spores per mL”. The problems of trueness and precision were also explained by a possible incomplete defrosting of the tubes and/or a lack of vortexing before analysis, resulting in insufficient homogenisation of the samples. Moreover, one laboratory indicated that diluting the most heavily loaded samples would have improved the precision of its results and should have been carried out. However, it should be noted that some participants obtained satisfactory results in the ILC without diluting the samples. Although no reason was specifically determined, a confusion between *Nosema* spp. spores and refractive artefacts could explain the anomaly in specificity, given that the laboratory had little experience in using this method. In 2018, to check the efficacy of the corrective actions carried out, the EURL sent a second panel of samples to the two laboratories interested in taking part in a new assay. The results were satisfactory, proving that the causes of the non-conformities had been resolved.

The ILC on *Nosema* spp. diagnosis by microscopy was the first assay involving the official laboratories of EU member states. Concerning the network of the 23 participating NRLs, test results showed global specificity of 98.6%, and global sensitivity of 100%. From a quantitative standpoint, trueness and precision were satisfactory for 92.8% and 93.5% of results respectively. These data demonstrated the performance of the network to provide the reliable analytical results that are essential to ensuring the quality of surveillance and study data. Although the analytical methods used by the NRLs were based on the same principle set out in the OIE Terrestrial Manual, they implemented different

technical procedures for preparing microscopic slides and counting. The data collected did not reveal any apparent link between the diversity of procedures and the conformity of results.

The trial used crushed abdomen samples. It would not have been feasible to use whole bees as *Nosema* spp. infection in honeybees is heterogeneous between individuals and not experimentally controllable. However, using whole bees as inter-comparison samples would have made it possible to evaluate the analytical methods in their entirety, *i.e.* including the first stages of the method: sampling for analysis, bee preparation, buffer type, grinding process and filtration procedures. The data collected by the questionnaire revealed a wide diversity in practices that could also influence results. For instance, some laboratories do not use only bee abdomens but whole bees or digestive tracts only in their routine analyses (16% and 8% of the participants respectively). Moreover, around 40% of the laboratories do not filter the suspension before microscopic examination. The presence of a significant quantity of particles and artefacts (such as pollen or yeast present in the digestive tract) could have an impact on the visual detection and counting of spores. A harmonised approach is certainly necessary for these early stages of the method.

Finally, the survey found that most NRLs did not have a diagnosis threshold for the clinical disease of nosemosis (*i.e.* a *Nosema* spp. spore load suggestive of an overt infection). Even when this threshold did exist, it varied from one million to several million or nine million spores per bee for *N. apis* (20% of laboratories having a threshold for this species). In the case of *N. ceranae,* two laboratories had a diagnosis threshold of one million of spores per bee. A recent study conducted in North America (Canada, Ontario) found that high levels of *N. ceranae* infections were significantly associated with reduced bee populations and food stores in colonies, and indeed suggested a intervention threshold of one million of spores per bee (Emsen et al. 2020). Several laboratories also said that the clinical signs observed in apiaries were considered in the interpretation of results. Harmonising the way in which results are interpreted, taking account not only of the spore load, but also the clinical signs and associated field information (*e.g.* presence of other sources of stress), would consolidate diagnostic modalities for nosemosis in the long term.

To conclude, the results of the ILC on *Nosema* spp. spore detection and counting were satisfactory overall. However, it should be mentioned that the panel, consisting of ten samples (three negatives and three positives, including two in triplicate) was relatively small. Further investigations with larger sample sizes should be conducted to consolidate the results and the conclusions of this study. In addition, the small number of anomalies identified must be relativised in view of the significant measurement uncertainties of the visual counting method and the absence of a scientifically established threshold for the diagnosis of *N. apis* and *N. ceranae*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106183.

Funding

This project was supported by the European Commission, Directorate General for Health and Food Safety, Brussels.

Declaration of Competing interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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