PROFICIENCY TEST METHODOLOGY

An inter-laboratory comparison of molecular methods for the identification of *Nosema* species in honeybee samples

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PROFICIENCY TEST METHODOLOGY

Abstract

To evaluate the performance of the molecular methods used by national reference laboratories (NRLs) for the identification of *Nosema* species in bee samples, an inter-laboratory comparison (ILC) was organised in 2015. A total of 20 EU NRLs and 1 non-European NRL participated in this ILC. The specificity of the methods was tested on various *Nosema* species: *Nosema apis*, *Nosema ceranae* and *Nosema bombi*. The test panel of samples provided to the laboratories contained 17 suspensions of crushed abdomens from naturally and artificially infected honeybees and bumblebees. In addition, data on the routine methods used by the participating laboratories were collected in an online survey, covering all the steps involved in DNA extraction and PCR. Our analysis showed that the 21 NRLs use 21 different protocols, each presenting variations from the DNA extraction step to the PCR step. The results of this ILC indicate that 48% of the participating laboratories returned the expected results. Considering the 21 different methods used, 57% of participating laboratories provided satisfactory results with regard to sensitivity, and 72% with regard to specificity. The results of this ILC clearly highlight the need for improved harmonisation of molecular *Nosema* identification methods.

Introduction

Two microsporidian (fungus) species have been described in honeybees: *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Fries *et al.*, 1996). The parasite multiplies in the epithelial cells of the posterior region of the honeybee ventriculus (mid-gut), leading to host cell burst and the release of a multitude of spores. The spores then infect other neighbouring cells, where they multiply, or are eliminated with the faeces. *N. apis* is a parasite of the European honeybee, *Apis mellifera*; while *N. ceranae* was originally a parasite of the Asian honeybee, *Apis cerana* (Fries *et al.*, 1996). However, in the last two decades, *N. ceranae* has been detected in several geographically distant populations of *A. mellifera* in Europe, South America, North America and Asia (Chaimanee *et al.*, 2011; Chen *et al.*, 2008; Higes *et al.*, 2006; Huang *et al.*, 2007; Li *et al.*, 2012). It is not known when or where *N. ceranae* first infected European honeybees, but it has been present in Europe for at least two decades (Botías *et al.*, 2012). Today, *N. ceranae* is more frequently found in European honeybee colonies than *N. apis*, at least in some regions of Europe (Klee *et al.*, 2007; Paxton *et al.*, 2007). Unlike *N. apis* infection, the pathogenic effects of *N. ceranae* on colonies of *A. mellifera* are not very well known. *N. ceranae* may be involved in colony weakening associated with other sources of stress (Alaux *et al.*, 2010; Doublet *et al.*, 2015a; Doublet *et al.*, 2015b; Vidau *et al.*, 2011; Zheng *et al.*, 2015).

Nosema infection is highly contagious and spreads easily through the exchange of spores during feeding (trophallaxis) or comb-cleaning. Beekeeping equipment, contaminated honey stores and infected water also play a role in the transmission of the disease. In faeces, *N. apis* spores are viable for up to several months depending on weather conditions (Fenoy *et al.*, 2009; Sánchez Collado *et al.*, 2014). While the duration of viability of *N. ceranae* spores is unknown, it was shown that freezing significantly reduces the viability and infectivity of *N. ceranae* (Fries and Forsgren, 2009).

Nosema spp. are not covered by EU regulations or by the World Organisation for Animal Health (OIE) classification. However, the detection and identification of *Nosema* is described in the OIE terrestrial manual (World Organisation for Animal Health (OIE), 2013). Moreover, *N. apis* infection is classified as a category 1 health hazard in the French Rural Code (Article D. 223-21).

Although the presence of *Nosema* spores in a bee sample can be detected by optical microscopy, it is very difficult to differentiate the two *Nosema* species. This can, nonetheless, be done by transmission electron microscopy (Fries, 1989; Fries *et al.*, 1996). However, only molecular methods can reliably distinguish the two species. In the past few years, several PCR-

PROFICIENCY TEST METHODOLOGY

based methods (conventional and real-time PCRs) have been developed and implemented to identify *Nosema* species (Bourgeois *et al.*, 2010; Burgher-Maclellan *et al.*, 2010; Chen *et al.*, 2009; Erler *et al.*, 2011; Forsgren and Fries, 2010; Gisder and Genersch, 2013; Higes *et al.*, 2006; Klee *et al.*, 2007; Martin-Hernandez *et al.*, 2007; Traver and Fell, 2011). Most methods are based on PCR amplification and target the 16S rRNA gene using appropriate species-specific PCR primer pairs, except the duplex PCR method described by Gisder and Genersch (2013), which targets the DNA-dependent RNA-polymerase II gene. Based on the conventional multiplex PCR method (Martin-Hernandez *et al.*, 2007), and to avoid potential lack of sensitivity due to multiplex reactions, the EURL has proposed two species-specific uniplex PCRs (Carletto *et al.*, 2013). In a context with multiple molecular methods available (list in the BEEBOOK paper by Fries *et al.*, 2013), it became clear that a preliminary study was needed in EU laboratories to build an inventory of practices. The aim of this study was therefore to evaluate the performance of the molecular methods routinely used by the NRLs to identify *Nosema* spp. on a single sample panel. The sensitivity and specificity of the methods were also assessed.

Materials and methods

Participating laboratories

The European Union Reference Laboratory (EURL) for honeybee health (ANSES, Sophia-Antipolis Laboratory, France) organised an inter-laboratory ring trial in 2015. In total, 21 reference laboratories for honeybee health participated in the trial, 20 of which are EU member state NRLs, and 1 non-European NRL. In order to ensure the confidentiality of the results, each participating laboratory was assigned a random code number (lab1 to lab12, lab19 to lab27).

Reference method used by the EURL

The reference method for DNA extraction was as follows: briefly, a pool of 10 crushed bee abdomens was prepared, filtered through two layers of gauze, and washed twice with distilled water. After counting the spores, 80 µl of the solution were used for DNA extraction with the High Pure PCR Template Preparation Kit (Roche Diagnostics). Extracted DNA was resuspended in 200 µl elution buffer, according to the manufacturer's recommendations, and stored at -20°C until further analysis (used as a template in the PCR).

The conditions of the reference PCR method were as follows: 25 µl reaction mixture containing 1 U Platinum Taq DNA polymerase (Invitrogen), 0.4 µM each primer, 0.4 mM dNTPs and H_2O for a reaction volume of 20 µl, and 5 µl of DNA extracted from tested samples. For PCR reactions, an Eppendorf Mastercycler® Nexus ThermoCycler was used with the following cycling conditions: an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 30 s at 94°C, 30 s at 62°C and 30 s at 72°C, and a final extension of 7 min at 72°C. The two *Nosema* species (*N. apis* and *N. ceranae*) were differentiated using the primers described in Martin-Hernandez *et al.* (2007) in two separate uniplex PCR reactions (218 MITOC FOR, 218 MITOC REV, 321 *Apis* FOR, 321 *Apis* REV).

Sample selection and panel composition

The samples originated from the reference collection at the ANSES Sophia-Antipolis laboratory. The presence and quantity of *Nosema* spp. spores were determined based on microscopic counts (World Organisation for Animal Health (OIE), 2008). The presence/absence of *N. apis* and *N. ceranae* in the samples was determined using the molecular identification method of *Nosema* species described above, *i.e.* the reference PCR method, followed by sequencing of the amplicons produced.

All test panels were composed of samples originating from the same batches of crushed bee

PROFICIENCY TEST METHODOLOGY

samples (see “Production and control of test samples”). Each panel contained 17 samples for the ILC and included seven negative *N. apis* and *N. ceranae* samples, four positive *N. apis* samples, four positive *N. ceranae* samples, and two positive samples containing both *N. apis* and *N. ceranae* (Table 1). In addition, one positive *N. ceranae* sample or one sample free from *Nosema* spp. was added as a lure for each laboratory.

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

Sample	Status	Nosema species	Spores/ml count	Criterion evaluated
Na1	Positive	N. apis	3.20E+06	Sensitivity
Na2, 3, 4 (=Na1 diluted 1:5)	Positive	N. apis	6.40E+05*	Sensitivity
Nc1	Positive	N. ceranae	1.10E+05	Sensitivity
Nc2	Positive	N. ceranae	4.10E+06	Sensitivity
Nc3	Positive	N. ceranae	1.42E+06	Sensitivity
Nc4 (=Nc2 diluted 1:20)	Positive	N. ceranae	2.05E+05*	Sensitivity
Na/Nc1	Positive	N. apis / N. ceranae	6.40E+05 / 2.05E+06	Sensitivity
Na/Nc2	Positive	N. apis / N. ceranae	6.40E+05 / 7.00E+05	Sensitivity
AS1	Negative	-	-	Specificity
AS2	Negative	-	-	Specificity
AS3	Negative	-	-	Specificity
AS4	Negative	-	-	Specificity
AS5	Negative	-	-	Specificity
NB1	Negative	N. bombi	8.40E+06	Specificity
NB2	Negative	N. bombi	1.46E+07	Specificity

*Theoretical count: Na: *N. apis*; Nc: *N. ceranae*; AS: negative sample; NB: *N. bombi*.

Five out of seven negative samples were prepared from the abdomens of healthy bees from the EURL experimental apiary or from diagnostic samples. The two remaining negative samples contained *N. bombi* spores prepared from infected bumblebee abdomens provided by the Dutch NRL.

One positive *N. apis* sample was prepared from the abdomens of honeybees that were experimentally infected. The other three samples were prepared from a dilution of this positive sample. The four positive *N. ceranae* samples were prepared using abdomens of honeybees naturally infected with *N. ceranae*. The two positive samples containing both *N. apis* and *N. ceranae* were prepared by pooling a positive *N. ceranae* sample with positive *N. apis* sample.

Production and control of test samples

Abdomens from bee samples were crushed in water (1 individual in 1 ml), and the suspension was filtered through two layers of gauze. The suspension was then centrifuged at 800 g for 6 min. The supernatant was removed and 1 ml/bee of water was added to the pellet. The amount of spores in the different samples varied from 3 to 180 times the limit of detection of the accredited EURL method. Each batch of crushed bee samples was divided into 200 µl aliquots (about 80 tubes per batch were prepared).

All batches were stored at -20°C until they were shipped to the participating laboratories. All batches of test samples were controlled throughout their preparation using the reference me-

PROFICIENCY TEST METHODOLOGY

thod. These tests were carried out according to the experimental plans indicated in Appendix B of the NF EN ISO 13528 Standard "Statistical methods for use in proficiency testing by inter-laboratory comparisons" (ISO 13528:2015). Homogeneity tests were performed for all batches of test samples: for each batch, homogeneity was tested by a duplicate analysis on 10 samples randomly selected from the batches of positive test samples and by a single analysis on 10 samples randomly selected from the batches of negative test samples, ensuring 20 results per batch of positive test samples and 10 results per batch of negative test samples.

The stability of the samples was tested using a duplicate analysis on three tubes randomly chosen among positive test samples, *i.e.* six results per sample. Three tubes from each test panel of samples (randomly chosen from each panel ready to be sent) were placed at room temperature. Stability was assessed on day 0 (data obtained in the homogeneity study) and on day 16, maximum date of receipt and analysis of the samples for the participating laboratories. The results of stability testing on day 16 were compared with those from the homogeneity tests (day 0).

Study design

The ILC was organised in compliance with the quality requirements described in ISO/IEC 17025 and ISO/IEC 17043 (ISO/IEC 17025:2005; ISO/IEC 17043:2010). The organising laboratory is accredited for the PCR method used to identify *N. apis* and *N. ceranae*. The samples were packed and transported between the EURL and the NRLs in compliance with UN3373 regulations (Biological Substance, Category B).

The participating laboratories received the samples with their laboratory code indicated on each sample. After receipt of the package, the laboratories stored the samples at -20°C until analysis and sent their results back within 15 days. Laboratories were required to report the results qualitatively (*N. apis* or *N. ceranae*, positive or negative). The specificity and sensitivity of the complete method (including DNA extraction and PCR assay) were evaluated.

Technical survey of techniques employed

This study was the first step to evaluate the level of harmonisation within the EU NRL network for the molecular identification of *Nosema* spp. The participating laboratories were asked to use their current assays on the test panel received from the EURL. The method employed had to be the complete method that the NRL routinely uses to identify *Nosema* species in samples. An online survey of the routinely used methods was sent to the laboratories. Questions were asked regarding each step of the routine procedure, from DNA extraction to the PCR assay. Tables 2 and 3 describe the protocols used by each laboratory and summarise the main differences in testing methods.

Analysis of results

Analytical results were sent by e-mail in a spreadsheet file to the EURL coordinator of the ILC for assessment. To evaluate the performance of the methods of the participating laboratories, specificity and sensitivity were calculated. Specificity was defined as the ability of the laboratory to report a negative result on a negative test sample. The expected specificity rate was 100% of negative results. Sensitivity was defined as the ability of the laboratory to determine the correct species of *Nosema* from a positive test sample. The expected sensitivity rate was 100% of positive results.

Statistical analysis

A kappa statistical analysis was used to estimate the level of agreement between the method used by the EURL and all the methods used by the NRLs. The qualitative criteria used for this value have been described elsewhere (Landis and Koch, 1977): < 0, no; 0-0.2, insignificant; 0.2-0.4, low; 0.4-0.6, moderate; 0.6-0.8, good; 0.8-1, very good or excellent. All statistical tests were performed in Statistica v. 8.0, and differences were considered significant when $p < 0.05$.

PROFICIENCY TEST METHODOLOGY

Results

Methods used in the ILC and survey results

All participating laboratories completed the online survey. Each laboratory followed its own routine DNA extraction procedures, which differed between laboratories. Table 2 summarises the main differences between the 21 extraction procedures. Of the 21 participating laboratories, 18 used commercial kits distributed by five suppliers: Qiagen (n=9), Roche (n=4), Promega (n=3), Macherey-Nagel (n=1) and MoBio Laboratories (n=1). The three remaining laboratories used an "in-house" method. DNA extraction was performed manually by 81% of the laboratories (17/21). Four laboratories used automated systems marketed by Qiagen (QIAcube, BioSpring 96) or Thermo ScientificTM (KingFisherTM). The reported volumes used for the DNA extraction varied from 50 µl to 2 ml, and the reported DNA elution volumes ranged from 40 µl to 200 µl (Table 2). Given that 200 µl of each test sample were provided for the ILC study, some laboratories needed to adapt their method. However, 38% of laboratories (8/21) extracted DNA directly from the 200 µl sample and nearly 48% of them (10/21) eluted DNA in 100 µl.

TABLE 2/ Details of the DNA extraction methods implemented by each participating laboratory.

Laboratory Code	Commercial kit	Method	Extraction volume	DNA elution volume
Lab1	Roche	manual	2 000 µl	100 µl
Lab2	no	manual	150 µl	500 µl
Lab3	no	manual	100 µl	100 µl
Lab4	Qiagen	manual	200 µl	200 µl
Lab5	Promega	manual	50 µl	40 µl
Lab6	Roche	manual	200 µl	50 µl
Lab7	Qiagen	manual	200 µl	100 µl
Lab8	MoBio	manual	250 µl	100 µl
Lab9	Qiagen	manual	80 µl	200 µl
Lab10	Macherey-Nagel	manual	60 µl	60 µl
Lab11	Qiagen	automated	200 µl	100 µl
Lab12	Qiagen	manual	200 µl	120 µl
Lab19	no	manual	200 µl	100 µl
Lab20	Roche	manual	80 µl	200 µl
Lab21	Qiagen	manual	400 µl	200 µl
Lab22	Promega	manual	200 µl	100 µl
Lab23	Qiagen	manual	100 µl	100 µl
Lab24	Roche	manual	150 µl	200 µl
Lab25	Qiagen	automated	50 µl	100 µl
Lab26	Qiagen	automated	200 µl	100 µl
Lab27	Promega	automated	100 µl	200 µl

Regarding the PCR step, eight different primer pairs, the majority of which are described in the literature, were used by the 21 laboratories targeting five different genes (Table 3). The 16S rRNA gene (also called SSU rRNA) was the most frequently used target. The other four targets used were the DNA-dependent RNA-polymerase II gene (also called RPB1), the 18S rRNA gene, another part of the rRNA gene, and the U97150/c1 gene. More than half of the laboratories (12/21) used the primers described in Martin-Hernandez *et al.* (2007). One la-

PROFICIENCY TEST METHODOLOGY

boratory used the PCR method recommended by the EURL, and three laboratories used the multiplex PCR described in the OIE Manual (2013).

More than 76% of the laboratories (16/21) used a conventional PCR method, with half using uniplex reactions and the other half multiplex reactions. The five remaining laboratories used uniplex or multiplex real-time PCR.

TABLE 3/ Details of PCR assays implemented by each participating laboratory.

Laboratory Code	PCR assays				
	Type of PCR	DNA sample volume	PCR volume	Target gene	Reference
Lab1	conventional / multiplex	2 µl	25 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab2	conventional / uniplex	1 µl	25 µl	18S rRNA	not published
Lab3	conventional / multiplex	2 µl	25 µl	16S rRNA	Fries (OIE 2013)
Lab4	conventional / multiplex	3 µl	25 µl	RNA pol	Gisder and Genersch (2013)
Lab5	conventional / uniplex	5 µl	25 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab6	conventional / multiplex	15 µl	50 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab7	conventional / multiplex	2.5 µl	25 µl	16S rRNA	Fries (OIE 2013)
Lab8	real-time / multiplex	2 µl	20 µl	rRNA	Bourgeois et al. (2010)
Lab9	real-time / uniplex	5 µl	25 µl	SSU rRNA	Chen et al. (2009)
Lab10	conventional / uniplex	3 µl	25 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab11	real-time / multiplex	5 µl	25 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab12	conventional / uniplex	10 µl	50 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab19	conventional / uniplex	5 µl	25 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab20	conventional / uniplex	5 µl	25 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab21	conventional / multiplex	5 µl	50 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab22	conventional / multiplex	2.5 µl	25 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab23	conventional / uniplex	5 µl	25 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab24	conventional / uniplex	5 µl	50 µl	16S rRNA	Martin-Hernandez et al. (2007)
Lab25	conventional / multiplex	5 µl	25 µl	16S rRNA	Fries (OIE 2013)
Lab26	real-time / uniplex	2 µl	20 µl	16S rRNA	Forsgren, E., Fries, I. (2010)
Lab27	real-time / uniplex	1 µl	25 µl	U97150 / c1	not published

Sensitivity and specificity

The results obtained for the sensitivity test (positive test samples) by the participating laboratories are shown in Table 4. Overall, 84 results for each *Nosema* species were expected (4 per participating laboratory). Including all positive samples, 96% of the *N. apis* test samples (81/84) were identified and 90% of the *N. ceranae* test samples (76/84) were identified. For 7 out of 168 tested positive samples (4%), a species other than the correct one was identified. For the samples in which both species were present, 42 results were expected. Three laboratories had negative results and eight identified only one species.

The proportion of correctly identified positive *N. apis* and *N. ceranae* samples was calculated. The sensitivity of the identification methods used by the laboratories ranged from 40% to 100%, with an overall result of 85.2% (Table 6). Of the 21 laboratories, 12 achieved the goal of 100% sensitivity (57.1%).

PROFICIENCY TEST METHODOLOGY

TABLE 4/ Sensitivity results for each participating laboratory based on the positive test samples (expected results) (see Table 1 for sample composition).

Laboratory Code	Results on positive samples									
	Na1	Na2	Na3	Na4	Nc1	Nc2	Nc3	Nc4	Na/Nc1	Na/Nc2
lab1	Na	Na	Na	Na	Nc	Nc	Negative	Negative	Na/Nc	Na/Nc
lab2	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab3	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Nc	Nc
lab4	Na	Na	Na	Na	Na/Nc	Nc	Na/Nc	Na/Nc	Na/Nc	Na/Nc
lab5	Na	Na/Nc	Na/Nc	Na	Nc	Nc	Nc	Negative	Negative	Nc
lab6	Na	Na	Na	Na	Negative	Nc	Negative	Negative	Negative	Na
lab7	Na/Nc	Na/Nc	Na	Na	Nc	Nc	Nc	Nc	Negative	Nc
lab8	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab9	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab10	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab11	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab12	Na	Na	Na	Na	Nc	Nc		Nc	Na/Nc	Na/Nc
lab19	Negative	Na	Negative	Negative	Nc	Negative	Negative	Nc	Na/Nc	Na
lab20	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab21	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab22	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab23	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab24	Na	Na	Na	Na	Nc	Nc	Nc	Negative	Na	Na
lab25	Na	Na	Na	Na	Nc	Nc	Nc	Nc	Na/Nc	Na/Nc
lab26	Na	Na	Na	Na	Nc	Nc		Nc	Na/Nc	Na/Nc
lab27	Na	Na	Na	Na	Nc	Nc		Negative	Na/Nc	Na/Nc

The results are expressed for each sample as Na (*N. apis* detected), Nc (*N. ceranae* detected), Na/Nc (*N. apis* and *N. ceranae* detected) or negative. Non-compliant results are highlighted in orange.

The specificity results (negative samples properly identified) are shown in Table 5. Overall, 147 negative results were expected (7 per participating laboratory). Considering the samples free of *Nosema* spores, 87% of the negative test samples (92/105) tested negative. Thirteen false-positive results were reported by five participants. *N. ceranae* and *N. apis* were falsely detected in 11 and 2 negative samples, respectively.

TABLE 5/ Specificity results for each participating laboratory on the negative test samples (expected results) (see Table 1 for sample composition).

Laboratory Code	Results on negative samples						
	AS1	AS2	AS3	AS4	AS5	NB1	NB2
lab1	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab2	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab3	Nc	Nc	Nc	Nc	Negative	Nc	Negative
lab4	Negative	Negative	Na	Na	Negative	Negative	Na
lab5	Negative	Negative	Nc	Negative	Negative	Nc	Negative
lab6	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab7	Nc	Nc	Nc	Nc	Nc	Negative	Negative

PROFICIENCY TEST METHODOLOGY

Laboratory Code	Results on negative samples						
	AS1	AS2	AS3	AS4	AS5	NB1	NB2
lab8	Negative	Negative	Negative	Negative	Negative	Nc	Nc
lab9	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab10	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab11	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab12	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab19	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab20	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab21	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab22	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab23	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab24	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab25	Negative	Negative	Negative	Negative	Negative	Negative	Negative
lab26	Negative	Negative	Negative	Nc	Negative	Nc	Nc
lab27	Negative	Negative	Negative	Negative	Negative	Negative	Negative

The results of PCR are expressed for each sample as Na (*N. apis* detected) or Nc (*N. ceranae* detected) or negative. Negative samples were free of *Nosema* spores or contained *N. bombi*. Non-compliant results are highlighted in orange.

Of the 42 samples containing *N. bombi*, 7 test results did not match the expected result. Importantly, five laboratories (lab3, lab4, lab5, lab8, and lab26) incorrectly identified *N. ceranae* and *N. apis* in *N. bombi* samples. Laboratory lab4 incorrectly identified *N. apis*; the 4 remaining laboratories incorrectly identified *N. ceranae* in *N. bombi* samples. One of these laboratories (lab8) reported the expected results for true-negative samples (samples free of *Nosema* spores) and the other 4 laboratories reported false-positives.

The specificity of the identification method used in the laboratories ranged from 28.6% to 100%, with an overall result of 86.4% (Table 6). Overall, 15 laboratories attained the expected 100% for specificity (71.4%).

TABLE 6/ Sensitivity and specificity rates attained by each participating laboratory.

Laboratory Code	Sensitivity[a]		Specificity[b]	
	(%)	95% confidence interval (%)	(%)	95% confidence interval (%)
lab1	80	49.0 - 94.3	100	64.6 - 100.0
lab2	100	72.2 - 100.0	100	64.6 - 100.0
lab3	80	49.0 - 94.3	28.6	8.2 - 64.1
lab4	70	39.7 - 89.2	57.1	25.0 - 84.2
lab5	50	23.7 - 76.3	71.4	35.9 - 91.8
lab6	50	23.7 - 76.3	100	64.6 - 100.0
lab7	60	31.3 - 83.2	28.6	8.2 - 64.1
lab8	100	72.2 - 100.0	71.4	35.9 - 91.8
lab9	100	72.2 - 100.0	100	64.6 - 100.0
lab10	100	72.2 - 100.0	100	64.6 - 100.0

PROFICIENCY TEST METHODOLOGY

Laboratory Code	Sensitivity[a]		Specificity[b]	
	(%)	95% confidence interval (%)	(%)	95% confidence interval (%)
lab11	100	72.2 - 100.0	100	64.6 - 100.0
lab12	100	72.2 - 100.0	100	64.6 - 100.0
lab19	40	16.8 - 68.7	100	64.6 - 100.0
lab20	100	72.2 - 100.0	100	64.6 - 100.0
lab21	100	72.2 - 100.0	100	64.6 - 100.0
lab22	100	72.2 - 100.0	100	64.6 - 100.0
lab23	100	72.2 - 100.0	100	64.6 - 100.0
lab24	70	39.7 - 89.2	100	64.6 - 100.0
lab25	100	72.2 - 100.0	100	64.6 - 100.0
lab26	100	72.2 - 100.0	57.1	25.0 - 84.2
lab27	90	59.6 - 98.2	100	64.6 - 100.0
Overall	85.2 (179/210)	79.8 - 89.4	86.4 (127/147)	79.9 - 91.0

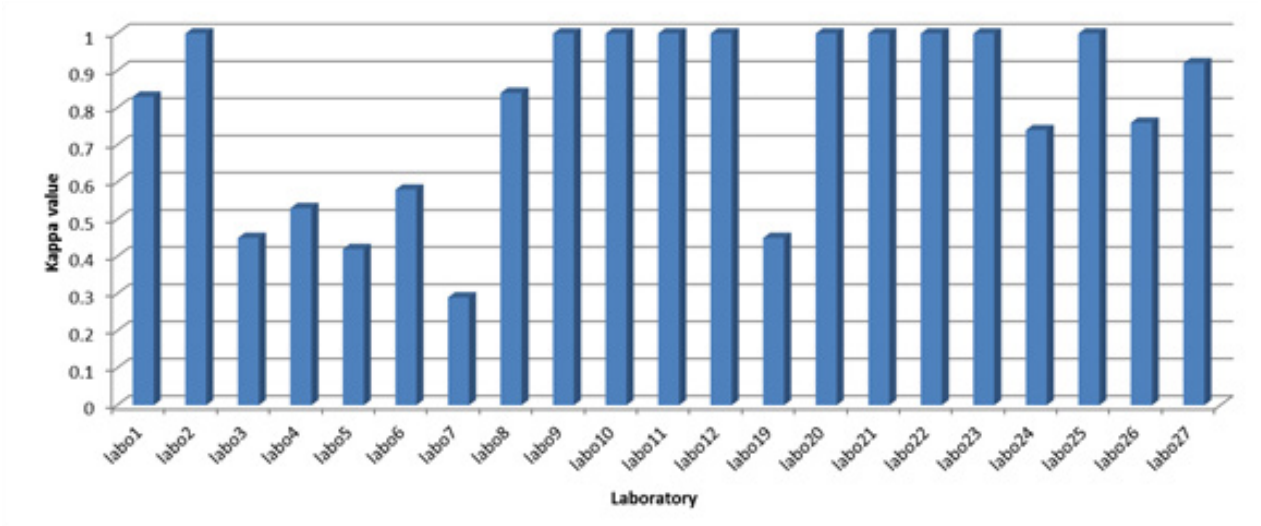
a The percentage of sensitivity was calculated from 10 samples (see Table 4).

b The percentage of specificity was calculated from 7 samples (see Table 5).

Performance of the methods used by the NRLs

Twenty-one different methods were used in this ILC: each of the 21 participating laboratories had its own method. Of the 21 protocols, 11 did not provide satisfactory results with regard to specificity and/or sensitivity (Table 6). The results of the kappa statistical analysis to evaluate the agreement of the NRL results with the results using the EURL reference method are shown in Figure 1. Most of the NRL results showed moderate to very good agreement with expected results. Very good agreement was obtained for 62% laboratories (13/21) and only one laboratory showed a low agreement (kappa value = 0.29).

FIGURE 1/ Level of agreement (kappa value) between the EURL PCR method and the methods used in the participating EU and non-European reference laboratories.



PROFICIENCY TEST METHODOLOGY

Discussion

Nosema species are frequent pathogens that may cause colony death, alone or associated with other factors such as other pathogens (Doublet *et al.*, 2015b; Zheng *et al.*, 2015) or pesticides (Doublet *et al.*, 2015a). *N. apis* was historically believed to be the only species infecting *A. mellifera* in Europe. However, for the past two decades, *N. ceranae* has been detected in European colonies of *A. mellifera* and seems to have replaced *N. apis* in some of the South European countries. Unlike *N. apis* infection, *N. ceranae* infection does not seem to lead to clear, visible symptoms. However, its impact as a cofactor is widely accepted. Therefore, the identification of *Nosema* species infecting bees is clearly important for disease management. Since the morphological characteristics of the two species are very similar, molecular tools have been developed. Several methods are available for diagnostic laboratories: conventional or real-time PCRs (Rivière *et al.*, 2013). The present study was organised to document and compare the performance of different methods implemented by the EU NRLs. This inter-laboratory comparison provided sensitivity and specificity data for these methods.

Although some comparative studies on different methods have been reported (Stevanovic *et al.*, 2010; Erler *et al.*, 2011), no comparative tests have been performed to date to evaluate the reliability of *Nosema* species identification within a network of reference laboratories. The present ILC involved 20 EU laboratories and 1 laboratory located outside the EU. The panel was designed to evaluate the specificity and the sensitivity of each laboratory's method. Comparison of the survey results on the techniques used revealed high variation in the protocols employed for DNA extraction and for the PCR assay.

Among the 21 participating laboratories, 10 (48%) obtained proficiency results in compliance with the expected specificity and sensitivity (100%). Regarding the extraction methods, 9 out of 10 satisfactory results were obtained with commercial kits of different brands and one was obtained using an in-house method. Three laboratories (lab1, lab8 and lab21) had to adapt their extraction method because the volume provided for testing was 1.25 to 10 times lower than that used routinely. This change may have had an impact on the limit of detection. However, only lab1 was unable to detect *N. ceranae* in two samples. Fifty percent of the 16 laboratories using conventional PCR achieved satisfactory results. The same result was observed for real-time PCR.

Among the unsatisfactory results, two involved only specificity, five only sensitivity and four both criteria. Out of the six laboratories that encountered specificity problems (lab3, lab4, lab5, lab7, lab8 and lab26), the detection of *N. ceranae* in negative samples (17/147) was more frequent than the detection of *N. apis* (3/147), which occurred for only one laboratory (lab4). The method used in this laboratory was a conventional uniplex PCR targeting the DNA-dependent RNA-polymerase II gene. False-positive results were shown for both types of negative samples (samples free of *Nosema* species and on *N. bombi*-positive samples) for four laboratories, although contamination problems cannot be ruled out. Regarding sensitivity, for the three laboratories that had to adapt the extraction volume used, only one (lab1) failed to identify two *N. ceranae* positive samples. Out of the nine laboratories that encountered sensitivity problems (lab1, lab3 to lab7, lab19, lab24 and lab27), seven had difficulties in identifying *N. apis* or *N. ceranae* in some samples. This indicates that the limit of detection of the methods used was probably higher than for the EURL reference method. Three laboratories with results indicating a lack of specificity (lab4, lab5 and lab7) detected an additional species in samples containing only one species. Regarding the samples containing both species, six laboratories were not able to detect both.

The EURL offered its help to the 11 laboratories that obtained non-satisfactory results in order to identify, analyse and discuss the discrepancies; 7 have since been in contact with the EURL. One laboratory mentioned errors when reporting the results in the spreadsheet (lab4), and another error in the PCR assay for some samples (lab5). Two laboratories (lab5 and lab6)

PROFICIENCY TEST METHODOLOGY

obtained the expected results after a second assay by using the same test. However, in one case, high background noise was observed (close to the targeted amplicon size) that could lead to misinterpretations (lab5).

The discrepancies in test results can be attributed to several causes. The parameters that may have an impact on sensitivity include, for example, extraction problems, extraction volume, PCR inhibition or competition between the two species in multiplex PCR. Regarding the specificity of the methods used, the factors that may influence the results include contamination during DNA extraction or during preparation of the PCR reaction mixture, the specificity of primers and PCR conditions. However, in the present study, a discordant result could not be clearly attributed to a single factor. The goal of this study was to collect information on the methods routinely used in NRLs and to evaluate their performance in the identification of *N. apis* and *N. ceranae*. Our results strongly indicate a need for standardisation to obtain a common level of proficiency. This is one of the main tasks of EURLs, which are mandated by the European Commission to ensure the development and use of high-quality analytical methods across the EU. Implementing ILCs is one tool to ensure the use of effective analytical methods.

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