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Editorial

Networking or not networking: that is not a question!

As this 2nd issue of 2017 is being published, the process of establishing EU Reference Laboratories (EURLs) in plant health as part of the implementation of the new EU Official Controls Regulation 2017/625 is well under way and the role of National Reference Laboratories (NRLs) will be strengthened. The changes for the network of official laboratories, NRLs and EURLs with the release of this new regulation were presented in the previous issue. These positive developments will nonetheless mean that EU plant pest diagnostic laboratories will face many challenges. In such context learning from others and networking will be essential. Reference laboratories in other sectors (*e.g.* animal health) are well established, and exchanges of experience will be essential even if the specificities of the sectors may differ in some respects. Euroreference helps build these bridges by bringing together contributions from animal health, plant health, and food and drinking water safety.

In this issue:

- Investigations on Q Fever NRL's interactions with diagnostic laboratories are reported.
- An inter-laboratory comparison of molecular methods for the identification of *Nosema* species in honeybee samples is presented. Such comparisons, *i.e.* proficiency testing (PT) and test performance studies (TPS), have become an essential aspect of laboratory practice in all areas and their use is increasing internationally, PTs being often required in the accreditation process.
- The need for harmonisation of Certificates of Analysis of active substances provided by manufacturers for veterinary drugs is discussed.
- An overview on the revision of ISO/IEC 17025 standard is presented: This is the reference standard for accreditation for the methods implemented as part of official analyses under the EU 2017/625 Regulation.
- Reliable and rapid diagnostic processes are essential to support inspection activities conducted by National Plant Protection Organizations (NPPOs), and to evaluate the efficacy of measures taken. How EPPO and Euphresco aim to ensure high quality, harmonised plant health diagnostics in the EPPO region is presented.
- In the plant health field, hundreds of pests (bacteria, fungi and chromista, insects and mites, nematodes, phytoplasmas, viruses and viroids, invasive plants) are regulated in EPPO countries. Consequently laboratories may potentially need to perform tests on thousands of pest/host matrices under accreditation. In such case a flexible scope is a must and exchange of experience and harmonisation are needed. Laboratories need to have access to well-characterised biological reference material for morphological identifications and the use for development, validation of tests and as a base for positive and negative controls. Reports of networking activities organised by EPPO in the last months on these two important topics for reference laboratories are described.

We hope you will enjoy reading these articles and that this will stimulate further exchanges on these important topics. Let's confirm networking as a 2018 New Year resolution and we encourage you to share your experience through *Euroreference*.

Françoise Petter and Madeleine McMullen



NETWORKING

A National Reference Laboratory's interactions with veterinary diagnostic laboratories: example of Q fever, an abortive disease in ruminants and a zoonosis

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Abstract

To improve the control of targeted pathogens affecting animal and plant health, National Reference Laboratories (NRLs) have been mandated by the French Ministry of Agriculture. Their role involves ensuring that first-line departmental diagnostic laboratories are proficient in analytical methods. The NRL for Q Fever (QF-NRL) has developed measures contributing to the strong performance of methods within each laboratory's environment and on the network scale (national surveillance, epidemiological investigation). Following a survey of all (mandated and non-mandated) laboratories, the QF-NRL reports on the interactions between both parties and their interests, and outlines some prospects. Overall, the tools and exchanges (reference materials, validations, adoptions, control charts, inter-laboratory tests) are valued and provide means for determining the performance level of analytical methods and for proactively committing to further improvements.

Keywords

- ★ accreditation
- ★ analytical method
- ★ Control chart
- ★ ELISA
- ★ inter-laboratory comparison
- ★ method adoption
- ★ method validation
- ★ PCR
- ★ Q fever
- ★ reference materials

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Introduction

Caused by the bacteria *Coxiella burnetii*, Q fever is both a zoonosis and one of the main abortive diseases in domestic ruminants (OIE, 2015). It occurs worldwide and most species in the animal kingdom can be infected (Duron *et al.*, 2015; Lang, 1990; OIE, 2015). Ruminants are the primary reservoir for the bacteria causing human infection. It is especially at parturition and during abortions that infected animals can shed large bacterial loads into the environment (*via* placentas, vaginal secretions, faeces) (EFSA, 2010; de Crémoux *et al.*, 2012; Joulie *et al.*, 2015). Forms of the bacteria can survive in the environment and be dispersed (dust, aerosols). Transmission occurs primarily by air. Human cases of Q fever are typically sporadic. However, clustered cases regularly occur, often in *a priori* naïve populations in urban and peri-urban areas. For example, from 2007 to 2010, the Netherlands experienced the largest Q fever epidemic ever identified (more than 4000 human cases reported). The acute epidemic was controlled by drastic veterinary measures, such as a ban on the breeding and slaughter of gestating females and breeders, but the development of chronic forms of Q fever in exposed humans remains a problem for the coming decades (Van Asseldonk *et al.*, 2013). Risk factors for transmission to human populations are not completely understood. While the reservoirs and high-risk periods are known, these factors are harder to grasp. The emergence of human cases probably results from a combination of several factors such as the ambient bacterial load, the virulence of strains, the naïve immune status of people exposed, and especially factors favouring airborne diffusion (outdoor parturition, building cleaning, farm topography, dry and windy weather, etc.) (EFSA, 2010). In France, Q fever is not a notifiable disease. The actual number of cases in which treatment is sought is not known. Nonetheless, at least 200 hospitalisations related to Q fever are recorded each year according to the French Public Health Agency (Cazorla *et al.*, 2013).

In 2009, during the first wave of appointments of National Reference Laboratories (NRLs) in the field of veterinary public health and plant protection (Ministerial Order of 7 December 2016), ANSES's Sophia Antipolis Laboratory obtained the mandate of NRL for Q fever (QF-NRL). A long-standing activity was thus formalised. In fact, for the past 30 years, this laboratory has been organising inter-laboratory proficiency tests (ILPTs) for a large number of analytical laboratories for serological testing methods (Figure 1). Between 2007 and 2011, the QF-NRL also coordinated inter-laboratory tests for four counterpart agencies in EU Member States, in order to compare the performance of methods used in the areas of serology (ELISA and complement fixation), PCR detection (conventional and real-time) and molecular typing. It thus provided French and foreign diagnostic laboratories with the required reference materials (RMs) for ELISA serology and molecular biology. In 2013, the Sophia Antipolis Laboratory was recognised as the OIE Reference Laboratory for Q fever. With regards to research, it contributes to the development of tools and knowledge in order to better understand the epidemiology of animal Q fever, improve the control of infection, and thereby enhance the protection of public health. In France, Q fever is currently classified as a Category 3 health hazard for animal species, which means that no general-interest measures or collective mobilisation are provided for under the regulations. However, a State Note proposes a local organisational framework for the veterinary authorities in the event of clustered human cases with an investigation protocol for ruminant holdings and integrated management measures. Moreover, Q fever has been included as a priority topic for the National Epidemiological Surveillance Platform for Animal Health (ESA Platform; www.platforme-esa.fr) with the aim of better understanding the status of this disease in France. A pilot programme, paired with the surveillance plan for brucellosis, was implemented in 10 départements for three years (Gache *et al.*, 2017). With the creation of a network of 10 mandated laboratories for this programme, the QF-NRL reinforced its reference missions involving the standardisation and harmonisation of basic diagnostic methods: PCR and ELISA serology (Rousset & de Crémoux, 2013).

The pilot programme on Q fever ended in August 2015. This experiment contributed to the



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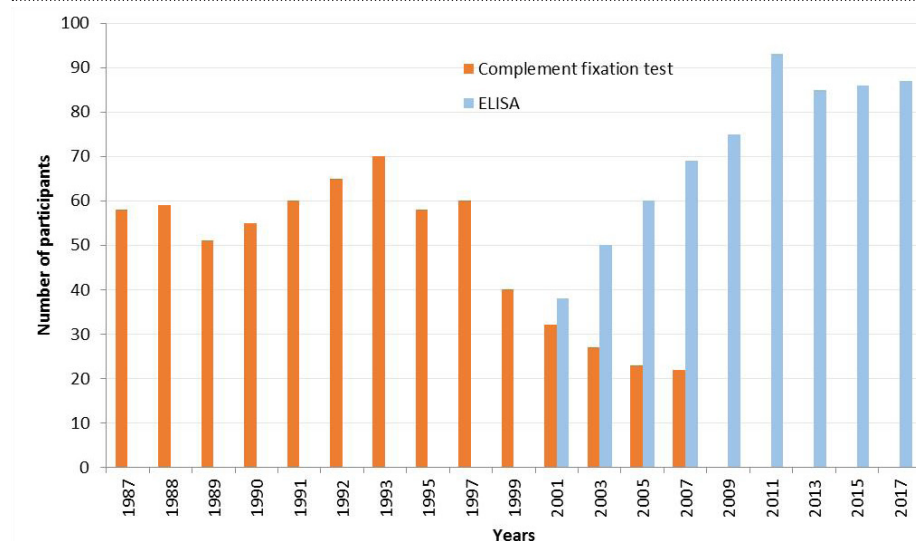
design of harmonised protocols for the differential diagnosis of abortions in ruminants and led to the deployment, in 2017, of a scheme called OSCAR (Observatory for Monitoring Causes of Abortions in Ruminants) aiming to collect, analyse and disseminate the results. Q fever is included in the differential diagnosis as a priority disease to be detected in both cattle and small ruminants. In this context, the QF-NRL undertook a survey of laboratories in the first quarter of 2016 involving a questionnaire with three sections: 1/ molecular biology and serology analyses, 2/ a proposed ILPT for PCR methods, and 3) the need for workshops with the NRL. The results of this survey were taken into account to draw up a review of the activities performed by the network of laboratories regarding Q fever and examine the NRL's actions to determine those to be improved and those to be extended to a larger number of laboratories.

Significance of the topic

Description of the network of mandated and recognised laboratories

The QF-NRL frequently addresses laboratories participating in ILPTs for serology (Figure 1), those developing new methods or amending the methods used (relative PCR vs quantitative PCR, other DNA extraction methods, modification of ELISA kits), and laboratories and kit manufacturers using reference materials (RMs). The deployment of the OSCAR scheme in 2017 may lead to an increase in requirements and related support actions, especially for PCR methods (Figure 2). The network thus comprises more than 60 public and private laboratories including 10 mandated laboratories in France and around 20 foreign laboratories (Figure 1). Non-mandated laboratories for which the NRL can have data or information on the quality of analyses are considered to be 'recognised' laboratories. This recognition of expertise by the NRL can favour a responsive mandate if necessary. The questionnaire was sent to the 83 French diagnostic laboratories. Despite a large number of questions (20, some of which were broken down), 44 questionnaires proved fully useable (53%, 44/83) while 10 were incomplete and could be used only for certain questions (65%, 54/83). The participation rate demonstrated laboratory interest in interactions with the dedicated NRL and indicated that the results were representative.

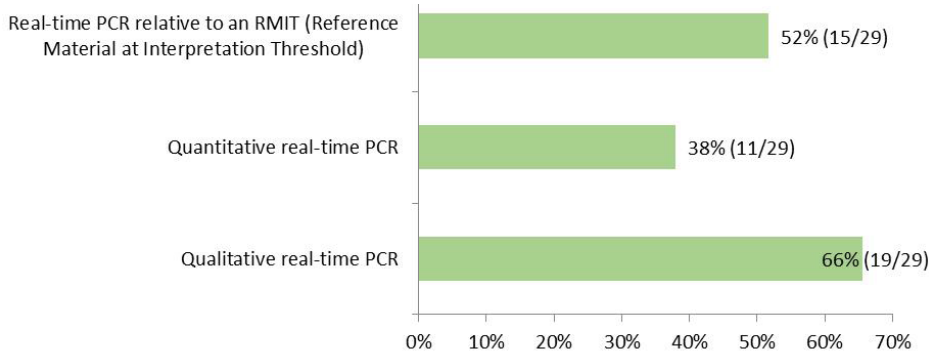
FIGURE 1/ Number of laboratories participating in inter-laboratory proficiency tests (ILPTs) for Q fever serology (complement fixation and ELISA) from 1987 to 2017.



Note: Unofficial tests for ELISA in 1997 and 1999; Transition between the two methods between 2001 and 2007 with four laboratories only for CF in 2007; Opening to foreign laboratories in 2009 (8, 21, 17, 22 and 22 from 2009 to 2017).

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FIGURE 2/ Proportion of laboratories using the various types of PCR.



Description of the Q fever analyses undertaken in French diagnostic laboratories

The survey confirmed that Q fever analyses are mainly performed in the context of diagnosing ruminant abortions. Serological testing from serum and PCR analyses from vaginal mucus or placentas are also used for certain epidemiological investigations. Serological testing from milk and PCR analyses from milk and faeces are seldom performed. The survey also highlighted a high proportion of laboratories using ELISA for serological testing (94%, 48/51), of which 82% participated in the ILPT organised by the NRL for this method (42/51). In addition, almost 80% of the respondents (42/54) perform or intend to implement PCR testing. In total, 72% of the laboratories can or wish to submit direct and indirect diagnostic results for Q fever (39/54).

Current contributions of the NRL

Support for the ISO 17025 accreditation process

The actions of the QF-NRL aim to ensure the quality of the results and interpretations produced by laboratories. One of the approaches consists in helping laboratories involved or wanting to be involved in an accreditation process. According to the survey, only 13% laboratories (7/54) are accredited for the two ranges of methods (ELISA and PCR). The main barriers to accreditation are an insufficient number of analyses, a lack of time, and/or budgetary constraints. The responses also show that ELISA methods are less often accredited (25% [12/48]) than PCR (45% [19/42]) (Figure 3).

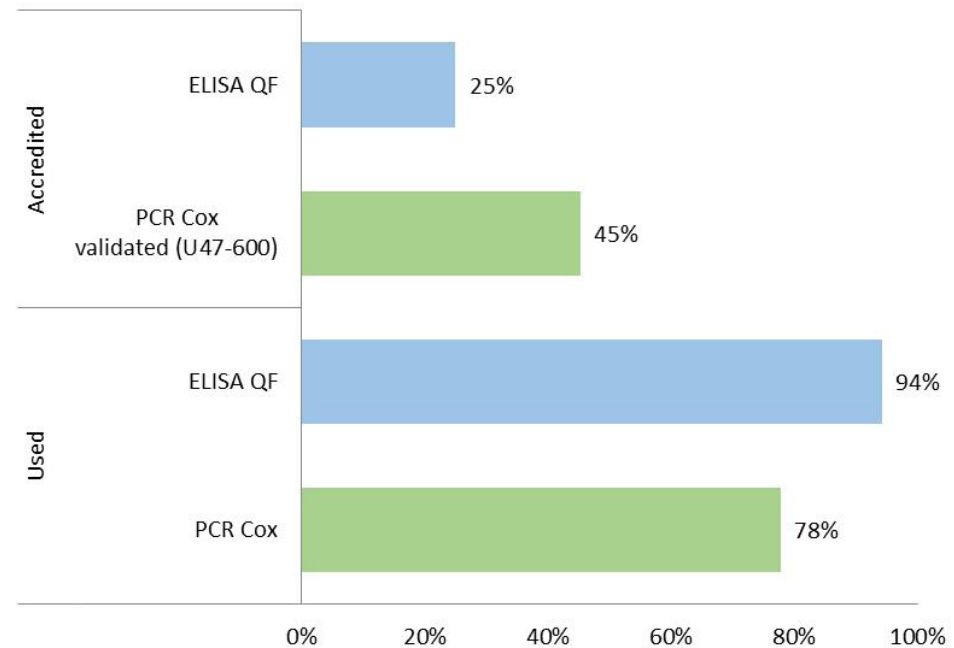
That said, ELISA methods have been used since the 1990s (Figure 1), whereas validated PCR methods have been implemented only more recently (Rousset *et al.*, 2012). The ELISA methods correspond to three commercial indirect ELISA kits, whose performance needs to be better evaluated (Rousset & de Crémoux, 2013; Horigan *et al.*, 2011; Emery *et al.*, 2012). Manufacturers wanting to validate these ELISA kits do not have a norm for serology like that for PCR; they also and most importantly do not have a sizeable collection of true negative and true positive sera (AFNOR, 2015; OIE, 2013). The logistics to obtain them are complicated and expensive for Q fever (complexity of experimental infections for ruminants in Biosafety Level 3 animal facilities, difficulties in qualifying the disease-free status of farms, etc.).

In the absence of a complete validation file, each diagnostic laboratory is required to undertake tests in order to confirm and provide evidence of the validity of the submitted results in relation to its own needs (confirmation file). A collection of characterised sera is required, even if it is more basic than for validation. Thus, there is a delay in terms of the standardisation of ELISA methods. Several actions are being pursued by the QF-NRL to better characterise the

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performance of existing serological methods: monitoring of batches of kits using a calibrating RM from the QF-NRL prior to the proposal of common acceptance criteria, and compared evaluation of ELISA kits by statistical modelling. PCR methods are also based on commercial kits.

FIGURE 3/ Proportion of accredited or unaccredited laboratories performing analytical methods for Q fever.



However, they were validated, according to French norm U47-600 and the performance stated by the QF-NRL, to be applied to the diagnosis of abortion in the framework of the 'pilot' programme deployed at the beginning of 2012 (Rousset & de Crémoux, 2013). The QF-NRL then supported the development of these methods (Rousset *et al.*, 2012). With duly validated methods, the laboratory only needs to verify their implementation, in its own environment, by referring to the criteria established to meet requirements for the diagnosis of abortion. Tests were first coordinated by the QF-NRL at the beginning of 2012, with the goal of mandating departmental laboratories from the pilot programme, and were then extended to other laboratories. The QF-NRL thus examined and certified the validation files of manufacturers and the verification test results of laboratories. Both of these probably influenced rapid changes in accreditation for PCR methods.

Needs for reference materials

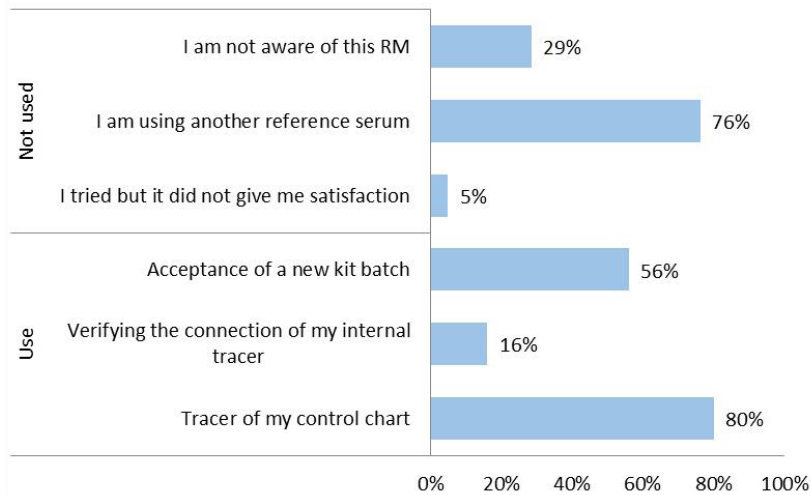
The survey's questions evaluated knowledge of RMs and their use by laboratories. Forty-five percent (19/42) of laboratories do not know about the RMs supplied for molecular biology methods; this figure is 13% (6/46) for that proposed for ELISA. Awareness-raising is still necessary. Nevertheless, many laboratories use or are planning to use the bacterial RM as a positive control and tracer for PCR methods (74%, 31/42). More than half of the laboratories already use the reference serum for ELISA analyses (54%, 25/46). Laboratories use it for one to three applications (Figure 4).

Eighty percent use it as a control chart tracer (20/25) and 56% for the acceptance of a new batch of kits (14/25). However, verification of the connection to an internal tracer seems to have less appeal (16%, 4/25). A high percentage of non-users use another reference serum (76%, 16/21). This serum may be an in-house material or be supplied by certain manufactu-

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users of ELISA kits or a group of laboratories. For PCR, to our knowledge, RMs are distributed only by the QF-NRL.

FIGURE 4/ Use of reference material (RM) for Q fever serology.



Changes in methods

Measurement uncertainty

The QF-NRL works to identify and encourage necessary changes in the methods used. For example, advice and quantitative data are specified in the latest ILPT reports on ELISA methods in order to promote the presentation of results with a level of uncertainty, inherent in any measurement method. This is a performance characteristic. The critical point is the measurement at the threshold level, which should be taken into account to identify results close to the threshold, to be distinguished from strictly positive or negative results. Knowledge of uncertainty at a method's threshold is enriched by inter-laboratory data from ILPTs and by initial verification data for the method or those from the control chart for a control calibrated to the threshold. The laboratories were surveyed regarding the reporting of results. The situation varies considerably between the two ranges of methods. For indirect diagnosis, 24% (11/46) specify measurement uncertainty on the results report. However, only one respondent, accredited for two types of PCR (quantitative and relative), gives this information for PCR results (2%, 1/42).

Relative (or semi-quantitative) PCR

Quantitative data were needed for the diagnosis of abortion in the pilot programme on Q fever. Real-time quantitative PCR (qPCR) was put into place but its financial cost is critical, especially due to the five quantification standards. The QF-NRL thus helped adapt the technique to make it more affordable. It recommended reporting results in relation to the diagnostic threshold for abortion, thus reducing the number of controls per series of analyses. The 'relative PCR in relation to a Reference material at interpretation threshold (RMIT)' type was taken into account in the revision of the PCR norm published in 2015 (AFNOR, 2015). Validations by manufacturers were undertaken and verified by the QF-NRL between mid-2016 and mid-2017. For the diagnosis of abortion, based on a clinical threshold, it proposed using either qPCR with a range or relative PCR in relation to an RMIT (rPCR). For the latter, the tracer is also the RMIT for the method. The number of controls for relative PCR is the same as for

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qualitative PCR and lower than for quantitative PCR. According to the survey results, at the beginning of 2016, the project for relative PCR interested half of the responding laboratories (21/42). Interest in this method is starting to increase, in particular among departmental laboratories volunteering to participate in the OSCAR scheme.

Future expectations

Inter-laboratory data for interactive monitoring

The QF-NRL wants to extend the use of similar inter-laboratory data to monitor the performance of PCR methods. The survey's results show strong laboratory interest in supporting these initiatives:

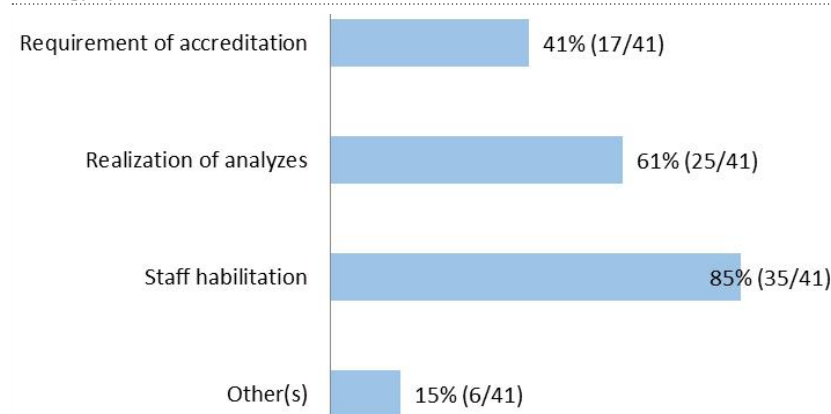
- 76% of laboratories (32/42) for initial verification results for the method according to a common experimental design,
- 74% of laboratories (31/42) for those of control charts based on a common assayed positive control (tracer/RMIT).

In the context of the network for the pilot programme, verification tests for methods, before their routine use, were a sort of ILPT. The collection of data from control charts enabled the responsive examination of problems encountered. It also made it possible to verify the reproducibility and accuracy of the qPCR results of the network of laboratories and thus determine whether they could be aggregated and used. These observations centralised by the QF-NRL seem useful to the laboratories, at least for the method's first two years of implementation.

Expectations for ILPTs

For the question asked about a first PCR-ILPT, 61% of laboratories are in favour (30/49). Both ILPTs were proposed for 2017, but the NRL will then need to organise ILPTs for Q fever on a biennial basis, alternating between ELISA and PCR. ILPTs contribute to the quality control process by enabling an external assessment. Participation is required by the French Accreditation Committee (COFRAC), but laboratory participation goes far beyond the accredited laboratories. Thus, every ILPT campaign for ELISA methods involves more than 60 French laboratories (Figure 1), showing that laboratories are concerned about verifying the quality of results, without necessarily wanting to be accredited for the method, and positioning themselves, in terms of results, in relation to other laboratories and the various ELISA methods available (Figure 5).

FIGURE 5/ Reasons for laboratory participation in inter-laboratory proficiency tests (ILPTs) for Q fever serology by ELISA.



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Need for a workshop with the NRL

The survey revealed an expectation involving workshops with the QF-NRL. For 83% (40/48) of the responding laboratories, an annual meeting is desirable; it should be open to kit manufacturers in particular.

Conclusion

The activity of NRLs is still relatively unknown. In France, NRLs are designated by Ministerial Order of the Minister of Agriculture. The tasks of NRLs and EURLs (European Union Reference Laboratories) are described in Regulation (EC) No 882/2004, recently replaced by Regulation (EU) No 625/2017 (Kremer & Carteau, 2017). With regards to veterinary laboratories, reference missions include the organisation of ILPTs, the preparation and distribution of RMs, method improvements, developments and validations, as well as scientific and technical monitoring, communication, and training. NRLs are also requested to provide support for the management of health crises in addition to expert appraisals for the health authorities. Reference activities are enhanced by knowledge of issues thanks to a series of interactions with various field stakeholders (analytical laboratories, veterinary practitioners, manufacturers of diagnostic kits and vaccines, health managers). The structured reference continuum also includes research work in collaboration with other scientific teams. Every theme generates specificities or special needs. A survey of 83 French laboratories conducted by the QF-NRL illustrated the various interactions and expectations of the network of over 60 'recognised' laboratories, including 10 mandated laboratories, which wants to be involved in better aligning its needs with the proposed reference activities. Overall, the survey showed that the responding laboratories are satisfied, and the normative framework driven and coordinated by the QF-NRL for the validation of PCR methods seems to provide a favourable context for accreditation in this area for diagnostic laboratories. For laboratories, the data and experiences shared via the QF-NRL are sources of information with which to respond to challenges and carry out improvements; overall, they build confidence in the quality of methods. For the QF-NRL, this knowledge of laboratories provides it with a responsive capacity to mandate new laboratories, or at the very least, recognise expertise for animal health managers, stakeholders in professional breeding sectors, and public health services.



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PROFICIENCY TEST METHODOLOGY

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

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Keywords

- ★ diagnosis
- ★ interlaboratory comparisons
- ★ microsporidia
- ★ *Nosema apis*
- ★ *Nosema ceranae*
- ★ PCR

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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-



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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 H₂O 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



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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	<i>N. apis</i>	3.20E+06	Sensitivity
Na2, 3, 4 (=Na1 diluted 1:5)	Positive	<i>N. apis</i>	6.40E+05*	Sensitivity
Nc1	Positive	<i>N. ceranae</i>	1.10E+05	Sensitivity
Nc2	Positive	<i>N. ceranae</i>	4.10E+06	Sensitivity
Nc3	Positive	<i>N. ceranae</i>	1.42E+06	Sensitivity
Nc4 (=Nc2 diluted 1:20)	Positive	<i>N. ceranae</i>	2.05E+05*	Sensitivity
Na/Nc1	Positive	<i>N. apis</i> / <i>N. ceranae</i>	6.40E+05 / 2.05E+06	Sensitivity
Na/Nc2	Positive	<i>N. apis</i> / <i>N. ceranae</i>	6.40E+05 / 7.00E+05	Sensitivity
AS1	Negative	-	-	Specificity
AS2	Negative	-	-	Specificity
AS3	Negative	-	-	Specificity
AS4	Negative	-	-	Specificity
AS5	Negative	-	-	Specificity
NB1	Negative	<i>N. bombi</i>	8.40E+06	Specificity
NB2	Negative	<i>N. bombi</i>	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-

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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 $p < 0.05$.



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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 Scientific™ (KingFisher™). 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-

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laboratory 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	Type of PCR	PCR assays				Reference
		DNA sample volume	PCR volume	Target gene		
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%).

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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

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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

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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

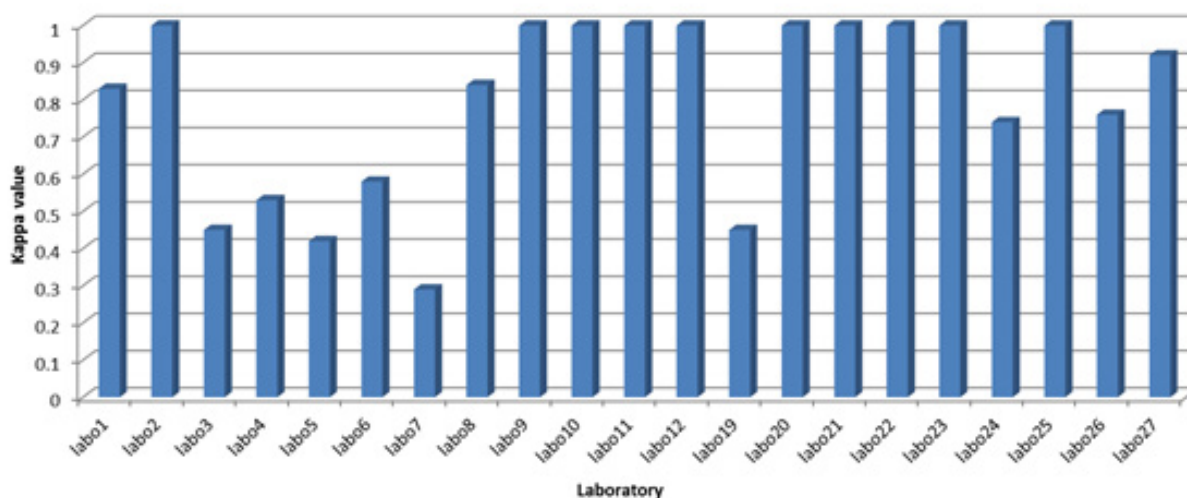
a The percentage of sensitivity was calculated from 10 samples (see Table 4).

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.



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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)



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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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Certificates of analysis: a challenge to interpret

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Abstract

The active pharmaceutical ingredient (API) in a medicinal product is the component that is responsible for the therapeutic effect. The personnel at the ANSES Fougères Laboratory routinely handle medicinal analytical standard and calculate their API content using the certificates of analysis provided by the manufacturer. However, meaningful data in these documents are not always easy to determine or may sometimes be absent: interpretation of these data can therefore result in assessment errors. Having identified this problem, the ANSES Laboratory has put forward an approach aimed at harmonising interpretation of certificates of analysis.

Keywords

- ★ Certificate of analysis
- ★ Data interpretation
- ★ Veterinary drugs
- ★ Active pharmaceutical ingredient

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Introduction

Medicinal products, both for human and veterinary use, contain a single drug substance or multiple drug substances, and excipients. The drug substance (DS), or active pharmaceutical ingredient (API), is the compound that underlies the pharmacological and therapeutic effects.

Given the various national and European mandates of the ANSES Fougères Laboratory as a reference laboratory in the area of veterinary medicinal products, its personnel handle medicinal analytical standards on a daily basis. This work is done as part of testing carried out during the development and validation of methods, official controls, proficiency testing, and research projects. It is therefore essential for the personnel to be able to calculate the amount of API in the analytical standards on the basis of the certificates of analysis (CoAs) supplied by the manufacturers.

However, CoAs are not all formulated in the same way and may be incomplete: interpretation of data may therefore lead to errors in evaluation.

The ANSES Fougères Laboratory has identified this problem and is therefore putting forward an approach to harmonise the interpretation of CoAs.

Certificates of analysis: essential but potentially problematic documents

The CoA is a document that defines the analytical standards (name, CAS number, molecular formula, molecular weight, etc.) and indicates the required specifications, including appearance, purity, solubility, and water content. It provides the results of the identification and quality testing performed by the manufacturer for a batch, on the basis of the criteria cited in a pharmacopoeia. Therefore, the CoA is an essential document for any user of an analytical standard to determine the API content.

There is, however, no standard certificate of analysis: each manufacturer presents the specifications of the analytical standard and the analytical results on the basis of their own criteria. It may happen that important indications for the calculation of the API content are missing or imprecise or, more rarely, incorrect. Interpreting CoAs may thus be a sensitive task, potentially leading to assessment errors by the operators, especially if the operator is not a chemist by initial training.

As an example, the ANSES Fougères Laboratory carried out a survey among two groups of operators regularly using antibiotics to test the interpretation of various “critical” CoAs, with the users asked to determine the API content in the sample analytical standards. The first group was made up of 14 users from a single laboratory (1 CoA for ampicillin sodium), the second included 23 users mostly working in different laboratories (3 different CoAs). In both cases, the conclusion was clear: whenever there were doubts on interpreting the CoA, or if the chemical substance was somewhat complex, the results of API content calculations were highly heterogeneous. For instance, in the first group of 14 users, 8 different values were obtained, from 873 to 939 µg ampicillin/mg of ampicillin sodium. For the second group of users, the variability of results was of greater concern, specifically for the values obtained from data in the CoA for spectinomycin dihydrochloride pentahydrate, where the API content values ranged from 363 to 878 µg API/mg of analytical standard.

These tests show unequivocally that there is a paradox in ensuring careful metrological oversight of measurement methods if we are less rigorous downstream.



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From the medicinal analytical standard to the active pharmaceutical ingredient content

The medicinal analytical standard is a powder containing a chemical substance, impurities, and often residual water. Indications in the CoA concerning the purity and the residual water content of the analytical standard can be used to calculate the chemical substance content. The indications concerning the identification of the chemical substance will be used to calculate the API content.

When the CoA is incomplete, calculating the API content of each analytical standard requires reference documentary resources that must be made available to the user.

These documents include, on the one hand, pharmacopoeias: they contain various monographs that indicate the criteria for purity of analytical standards and the analytical methods to use for testing purposes. These monographs are authoritative references for any substance or formula included in the pharmacopoeia. They provide a recognised framework and are regularly updated. On the other hand, the Merck Index has been a reference source for chemical products for more than 100 years and presents more than 10,000 monographs, including those for medicinal chemical substances. Testers can consult this Index to supplement the information indicated in the CoA when the available pharmacopoeias are not sufficient. Likewise, there are several databases for chemical compounds on the internet that are easily accessible and comprehensive.

Since difficulties in interpreting CoAs are found repeatedly, but almost never addressed in the literature (Brown, 2008), the ANSES Fougères Laboratory decided to take a pragmatic approach and create a guide for the interpretation of CoAs, within the framework of the laboratory's quality assurance system. This approach is described below, step by step.

Content of chemical substance in the analytical standard: selecting the value to use

In order to determine the chemical substance content of their analytical standards, manufacturers carry out an assay by referring to a reference standard. The results of this testing are expressed in the CoA in different ways: either in international units/mg (IU/mg), $\mu\text{g}/\text{mg}$, $\mu\text{g}/\text{ml}$, or as a percentage.

On CoAs, some analytical standards with antibiotic activity still have their content expressed in IU/mg (bacitracin, colistin, penicillins, sometimes streptomycin and tylosin, etc.), but it is not always easy, specifically for chemist users, to understand the notion of conversion coefficients IU/mg (originating from WHO standards). At the ANSES Fougères Laboratory, where most testing is performed in the framework of reference activities on veterinary medicinal product residues, the data of interest are related to the maximum residue limits (MRLs) and are therefore expressed in $\mu\text{g}/\text{kg}$. It was therefore decided within this context, excluding microbiological testing, that since CoAs for these antibiotics never indicate the level of impurities, this level would be considered to be equal to 0%, and therefore that the chemical substance content of the analytical standard is equal to 100%.

If, on the basis of the CoAs, the only quantitative indication that can be used to determine the chemical substance content of the analytical standard is purity, expressed in %, we need to assimilate this value to a chemical substance content in the analytical standard by default. For example, a CoA for cefquinome sulphate indicates: "Purity (HPLC): 99.4%". This figure indicates that the batch of analytical standard has a purity of 99.4% in cefquinome sulphate, *i.e.* 994 μg of cefquinome sulphate/mg of analytical standard.



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When the manufacturer indicates on the CoA a value in $\mu\text{g}/\text{mg}$, this complicates matters. In general, this value represents the content in API and not the content in chemical substance. To be sure, we need to check this information in a pharmacopoeia. In this example of a CoA for amikacin sulphate, it is the API content that is indicated: "Potency: 776 μg amikacin base/mg (anhydrous basis)". When we check the data in the United States Pharmacopoeia (USP), we find that the result of the assay must be between 691 and 806 μg amikacin/mg. Therefore, the value indicated clearly corresponds to the content in amikacin and not in amikacin sulphate.

More rarely, a value expressed in $\mu\text{g}/\text{mg}$ can reflect the chemical substance content. This is the case for tetracycline HCl, for example, since it is always mixed with its epimer, 4-epi-tetracycline. Checking in the USP shows that the content in tetracycline HCl, without taking into account the epimer, must not be less than 900 $\mu\text{g}/\text{mg}$.

On some CoAs, the chemical substance content is associated with the expression "as is". This means that the manufacturer assayed the chemical substance content in the analytical standard as it will be presented in its final packaging, without transformation (specifically loss on drying). As a result, the content indicated is the value that must be retained. We must not consider the water content of the analytical standard, even if this value is indicated elsewhere.

Some analytical standards are supplied in solution. In most cases, to prepare the solution, the supplier took into account the chemical substance content of the analytical standard; the chemical substance content of the solution is therefore 100%.

Sometimes, however, a CoA may indicate two values. It then becomes essential to refer to the pharmacopoeia to find out whether this is a value for chemical substance content or API content. An example from a CoA for ampicillin sodium shows the rationale to follow: "Assay (HPLC Weight%): 93.3% - Potency: 878 μg ampicillin/mg": 93.3% represents the % of ampicillin sodium (or chemical substance) in the analytical standard, 878 μg represents the API content (ampicillin) in the analytical standard (after verification in the USP35: from 845 to 988 μg ampicillin/mg). It is easier to retain directly the API content, *i.e.* the value 878 $\mu\text{g}/\text{mg}$.

If a CoA indicates two values for content expressed in the same way (*i.e.* both in % or both in $\mu\text{g}/\text{mg}$), we should always opt for the value obtained with the most specific method. If, for example, we can choose between a value obtained using a titrimetric method and a value obtained using a chromatographic method, the second value should be selected.

Understanding the water content of the analytical standard

On the CoAs, manufacturers mostly indicate the water content in analytical standard.

During the manufacturing process of an analytical standard, the chemical substance may be combined with one or more H_2O molecules or water of crystallisation. A molecule associated with water of crystallisation is known by the name hydrate: monohydrate, dihydrate, etc. This phenomenon is common, particularly during crystallisation of carboxylic acids and molecules presenting in this form such as beta-lactams (penicillins, cephalosporins, carbapenems), quinolones and fluoroquinolones.

Various methods (thermogravimetric, chemical, spectrometric) can be used to determine the water content. The Karl Fischer (KF) method is a titration technique which is based upon the oxidation of sulphur dioxide by iodine in a methanolic hydroxide solution. The KF method assays both residual water in the analytical standard (moisture) and water of crystallisation. When the method to determine the water content of an analytical standard in the form of hydrate (cephalexin hydrate, for example) is the KF method, we must be careful not to take into account the H_2O molecule when calculating the API/chemical substance ratio. The loss



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on drying method (gravimetry) is only used to assay residual water in the analytical standard.

On CoAs, water content is indicated but the method that was used to calculate this value is not given. For instance, we may see "Water": in most cases, we can find the method used in the pharmacopoeia. However, if this is not the case, we should choose the "residual water" option, unless we have the CoA for another batch of the analytical standard from the same manufacturer, with a similar water content.

In general, the water content is expressed in %, but in some cases it is expressed in mole of water/mole of chemical substance: we then need to think in terms of molecular weight. For example, the CoA of a batch of apramycin sulphate shows that there are 0.3 moles of water per mole of apramycin sulphate. This way of indicating the water content does not simplify the calculations. Particularly since, in this case, the sulphate content is also expressed in mole/mole.

In short, it is essential to know which technique was used to determine the water content. To do this, it is very important to refer to the pharmacopoeias. This is particularly critical for chemical substances in the form of hydrates.

Identification of the chemical substance: uncertainties

Knowing the identity of the chemical substance will serve to calculate its content in active pharmaceutical ingredient (API).

The identity of a chemical substance is indicated in part by its name but it is chiefly determined by a unique registration number from the Chemical Abstracts Service (CAS) database. The CAS attributes these numbers to all chemical products that have been described in scientific documentation. About 30 million compounds have been attributed a CAS number to date.

Most CoAs indicate this CAS number but in some cases it is not given. This can pose a problem in the event that a chemical substance described has different names. For example, cloxacillin sodium is also cloxacillin sodium monohydrate (CAS Number: 7081-44-9), not to be confused with cloxacillin sodium which is implied but not indicated as being "anhydrous" (CAS Number: 642-78-4). Other examples include pyrantel pamoate and pyrantel embonate, which are the same chemical substance (CAS Number: 22204-24-6), as well as sulphame-thazine, sulphadimidine and sulfadimerazine (CAS Number: 57-68-1). There are many similar examples showing the importance of knowing the CAS number. A chemical substance is also defined by its formula, whether semi-structural or molecular, and its molecular weight.

All these characteristics can be used to determine whether a chemical substance is a hydrate, and whether it is in salt form. The semi-structural formula tells us about the precise structure of the molecule, and the number of ions or elements it is made up of, which is not clear solely on the basis of the chemical substance's name. For instance oxytetracycline hydrochloride is made up of 1 "oxytetracycline" and 1 "hydrochloride", sisomicin sulphate is 2 "sisomycins" and 5 "sulphates", malachite green, 1 "malachite green cation" and 1 "chloride anion". Here again, there are multiple examples.

The active pharmaceutical ingredient content in the analytical standard: some complexity

Once the chemical substance and its chemical structure have been identified, it is easy to calculate the active pharmaceutical ingredient:chemical substance ratio (API:CS ratio). For



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example, for the chemical substance trimethoprim, there is 100% trimethoprim; for emamectin benzoate, all we need is the ratio of the molecular weight of emamectin versus that of emamectin benzoate. This yields a content: in this case 86.2%.

However, we will not need a calculation if the CoA indicates the chemical substance content of the analytical standard in µg/mg (excluding exceptions, see above). The calculation will nonetheless be more complex if the sulphate content is expressed in mole/mole.

We must always exercise caution when dealing with chemical substances that are in ionic form. For instance, in the case of nafcillin sodium, nafcillin "base" has lost an H⁺ proton but gained an Na⁺ proton. Therefore, the difference in molecular weight of nafcillin sodium versus nafcillin "base" is 22 instead of 23, a change we need to take account of when calculating the nafcillin/nafcillin sodium ratio.

Likewise, we must be careful concerning CoAs for chemical substances in hydrate form, where the water content was calculated using the KF method: we must not take the H₂O molecules into account in duplicate.

Once we have determined the purity (or content of the analytical standard in chemical substance), the water content and the API content (if necessary), these data are multiplied with one other to obtain the API content of the analytical standard. This operation is of course to be repeated with each change of manufactured batch of the analytical standard.

Example of calculating the API content based on the certificate of analysis

FIGURE 1/ Certificate of analysis for a batch of apramycin sulphate

Certificate of Analysis		
Product Name:	APRAMYCIN SULFATE SALT ~ 95 % TLC	
Product Number:	A2024	
Batch Number:	BCBP2820V	
CAS Number:	65710-07-8	
Formula:	$C_{21}H_{41}N_5O_{11} \cdot xH_2SO_4$	
Formula Weight:	539.58	
Storage Temperature:	2-8 C	
Quality Release Date:	09 JAN 2015	
TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	WHITE TO LIGHT YELLOW	LIGHT YELLOW
APPEARANCE (FORM)	POWDER	POWDER
PURITY (TLC AREA %)	APPROX. 95%	99 %
SOLUBILITY (COLOR)	COLORLESS TO LIGHT YELLOW	SLIGHTLY YELLOW
SOLUBILITY (TURBIDITY)	CLEAR (< 3.5 NTU)	CLEAR (<3.5 NTU)
SOLUBILITY (METHOD)	200 MG PLUS 8 ML OF WATER	200 MG PLUS 8 ML OF WATER
WATER	≤ 5 MOL/MOL	0.3 MOL/MOL
SULFATE	≤ 3 MOL/MOL BASED ON SULFUR	2.0 MOL/MOL

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The CoA used as an example (Figure 1) is for a batch of apramycin sulphate. The CoA is complete, but we must note that the molecular weight indicated is that of apramycin and not that of apramycin sulphate, which is important for the subsequent calculation.

Also, the CoA indicated that the purity of the analytical standard is equal to 99%, and that the water content is equal to 0.3 mole/mole (*i.e.* of apramycin sulphate) and its sulphate content 2 moles/mole (of apramycin sulphate).

Firstly, we need to calculate the water content of the analytical standard, expressed as a percentage. We must work with the molecular weight because the result is expressed in moles:

$$\frac{(0.3 \text{ mole water } (18.01 \times 0.3 = 5.4))}{1 \text{ mole apramycin } (539.58) + 2 \text{ moles sulfate } (2 \times 98.08 = 196.16) + 0.3 \text{ mole water } (5.4)} = \frac{5.4}{741.14} = 0.73\%$$

We then need a formula to obtain the API:CS ratio (active pharmaceutical ingredient:chemical substance ratio), also expressed as a percent (%):

$$\frac{1 \text{ mole apramycin } (539.58)}{1 \text{ mole apramycin } (539.58) + 2 \text{ moles sulfate } (2 \times 98.08 = 196.16)} = \frac{539.58}{735.74} = 73.3\%$$

As explained above, the data obtained are multiplied by one another to reach the API content of the analytical standard:

$$99\% (\text{purity}) \times 99.27\% (100\% - 0.73\% \text{ water}) \times 73.3\% \left(\text{ratio} \frac{\text{API}}{\text{CS}} \right) = 72.04\%$$

The API content of the analytical standard is equal to 72.04%, *i.e.* 720.4 µg active apramycin per mg of analytical standard.



The choice of the ANSES Fougères Laboratory: centralisation of calculations

To counter the risk of errors when interpreting CoAs, the ANSES Fougères Laboratory opted for a centralised approach: all the API contents of medicinal analytical standards used at the laboratory are evaluated by a reference person, assisted by deputies. When a CoA is received, and using a specific Excel spreadsheet and an internal interpretation guide for CoAs, which is constantly updated, the reference person carries out the necessary calculations. This person then records the API content of the analytical standard in the Laboratory Information Management System (LIMS) as a unique and traceable value that is accessible to all users of the analytical standard. The deputies, who are trained in performing these calculations, check the data and the record in succession, which also has the effect of ensuring that their skills are maintained.

Conclusion

The CoA is an essential document for users of medicinal analytical standards because it contains key data to calculate the API content. However, these data are not always easy to determine and/or interpret: sometimes, they may be missing or imprecise. To avoid errors in interpretation, which are possible among different users, the ANSES Fougères Laboratory recommends a standardised approach with data processing referred only to trained personnel who can consult a guide on the interpretation of CoAs. In this way, for each batch of medicinal

METHOD DEVELOPMENT

analytical standard, a single value for API content that is verified and traceable is available to all laboratory personnel.

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Revision of the ISO/IEC 17025 standard

Olivier Pierson^{1*}

A new version of the ISO/IEC 17025 standard, "General requirements for the competence of testing and calibration laboratories", will be published before the end of 2017. Since 1999, this document has been the reference for the accreditation of tens of thousands of laboratories around the world, but it is also used by an even greater number of industrial or research laboratories (or their customers) to benchmark their measurement and testing practices.

The standard covers all the activities for characterising samples or media through measurements or experimental observations, including where appropriate, sampling, the development of methods or the publishing of opinions and interpretations concerning the results.

The new version makes no changes to the standard's scope: it covers all types of tests and all types of laboratories, irrespective of their size or status. Nevertheless, the standard can now be used to determine the competence of bodies that only carry out sampling, if the sampling is associated with subsequent testing or calibration. It will include the possibility of writing reports on the sampling, thus improving control of the different potential subcontracting systems between samplers and testing laboratories.

Keywords

★ ISO/IEC standard

★ Revision

★ Quality

★ Testing and calibration Laboratories

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The basic principles of the standard remain unchanged:

- The selection, or possibly the validation of testing methods that are relevant and appropriate for the customer's needs;
- The reproducibility of results, based on the concepts of metrological traceability and measurement uncertainty, as well as on the qualification of the resources implemented;
- The ability to trace and control the laboratory activities, through a management system that includes the implementation of continuous improvement.

It was considered necessary to revise the current version, however.

Since 2005, when the standard was last updated, many terms and documents cited in the standard have evolved, thus making an update necessary. More fundamentally, analysis techniques, information systems, and procedures for transmitting results now offer opportunities that deserve to be taken into consideration. This is why a section has been introduced (among others) concerning information systems and the concept of reference data (commonly used in the fields of spectrometry or molecular biology). The format and the support medium for the information have been left as free as possible, including for the report itself.

As in 2005, the experts responsible for the revision have paid close attention to compatibility between ISO/IEC 17025 and ISO 9001. This is important, because many laboratories (including industrial laboratories) also seek ISO 9001 certification, because many quality tools and concepts are developed in an ISO 9001 environment, but especially because most laboratory customers are certified and expect their suppliers to be similarly organised.

The seven principles underlying ISO 9001:2015 have all been taken into account in the new version of ISO/IEC 17025. Some sections, such as the one relating to internal audits, have been closely aligned with ISO 9001. For the first time in the world of accreditation, the "risks and opportunities" approach has been incorporated, which should make it easier to scale implementation of the requirements according to the specific context of each laboratory. The process approach has been applied – strictly within the meaning of ISO 9001 – for activities involving sampling, testing or calibration, thus opening up many opportunities for laboratories to be integrated in a process-based management system. A notable result of this effort to reconcile the two reference standards is that ISO/IEC 17025 explicitly mentions that a laboratory conforming to ISO 9001 meets all the requirements of ISO 17025 concerning management systems.

Lastly, the general structure of the ISO/IEC 17025 standard has changed: it is now aligned with the structure of the recent standards for conformity assessment (including ISO 17020, for inspection activities). Here again, this choice reduces the effort required by organisations concerned by several such reference standards, and also presents a more pragmatic breakdown of requirements between resources, execution and management, better adapted for applying requirements in different contexts: industry, research, etc.

Plenty of good reasons, therefore, which explain the great interest aroused by the ISO/IEC 17025 standard (more than 2000 comments on average during each consultation) and a final 99% vote in favour among the Member States of the ISO committee concerned!

For reference laboratories, this new version will not introduce any mandatory fundamental changes, but may give more opportunities to take into account the specificity of their missions and their context, in particular by implementing the risk approach, digitising the results for data collection systems, and introducing the concept of reference data. The structure of the standard and the introduction of the process approach should also facilitate the management of other activities (training, proficiency testing, research) in the framework of a given management system.



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EPPO and Euphresco: how to ensure high quality, harmonised plant health diagnostics in the EPPO region

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Keywords

- ★ diagnostics
- ★ EPPO
- ★ Euphresco network
- ★ harmonisation
- ★ plant health

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Introduction

Reliable and rapid diagnostic processes are essential to support inspection activities conducted by National Plant Protection Organisations (NPPOs) in the framework of their official mandate, and to evaluate the efficacy of measures taken. Official controls aim to prevent or reduce the risk of introducing new pests through the agri-food trade and to protect consumer interests. The reliability and consistency of these controls contribute to effective trade. In this context, validated and internationally accepted diagnostic protocols are of the utmost importance as they support the harmonisation of detection and identification procedures worldwide, and contribute to greater transparency and comparability in the diagnosis of regulated pests (Petter *et al.*, 2007; Petter *et al.*, 2008).

Since 1998, the European and Mediterranean Plant Protection Organization (EPPO) has been supporting the harmonisation of diagnostic methods for regulated pests in the EPPO region through the development of technical [standards](#). As of September 2017, 130 diagnostic protocols have been approved by the National Plant Protection Organisations of the EPPO Member States. These protocols are developed by experts nominated by member countries. The EPPO Standards provide the guidance necessary for a pest to be detected and positively identified by an expert (*i.e.* an entomologist, mycologist, virologist, bacteriologist, nematologist, weed scientist, or molecular biologist), and cover different biological principles on which the tests used in different countries are based. For many years, the approach to diagnostic protocols has mostly been to include those tests that were already performed successfully in different laboratories, with validation data readily available from these laboratories. Since the establishment of the Euphresco network, a complementary approach has been followed: the need for the development and validation of new tests, or for the production of validation data for existing tests, is identified and addressed through research before drafting or revising a diagnostic protocol.

What is Euphresco?

In order to increase active collaboration among the organisations involved in plant health research activities at the national and regional levels, Euphresco (European Phytosanitary Research Coordination, www.euphresco.net) was established in 2006 and funded by the EU as an ERA-NET project. Euphresco has subsequently evolved into a self-sustaining international network hosted by EPPO. The benefits of this coordination are multiple (Giovani *et al.*, 2015; Giovani, 2017). By fostering collaboration at the research level, Euphresco enables researchers to work on common problems and contributes to the adoption of common standards and practices in research activities, including those with diagnostic aims. From the research area, these common approaches can spread to more applied activities, contributing to their harmonisation.

Every year, Euphresco members identify research priorities to be addressed through transnational collaboration. Many research projects have been funded with the aim of developing new tests for the detection and identification of pests, validating these tests or evaluating the proficiency of laboratories. Table 1 lists the most recent projects focussing on different diagnostic aspects.

Examples of the benefits of Euphresco projects

Close links have been established between Euphresco projects and EPPO activities in the field of diagnostic standardisation and these links are being developed further.

The Euphresco network brings together about 70 organisations, including all National Plant Protection Organisations (NPPOs) that are members of the EPPO. As such, they can participate in identifying the research priorities to be funded each year. Any questions arising from



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their routine activities can be addressed rapidly through international research collaborations that will provide scientific evidence to support the work of NPPOs. The outputs of Euphresco research projects provide valuable information for the development of EPPO regional Standards. Thus, the project 'Development and validation of innovative diagnostic tools for the detection of *Erwinia amylovora*', the causal agent of fire blight, provided validation data on tests for diagnosis in symptomatic and asymptomatic plant material to be used for the revision of EPPO Standard PM 7/20. Given the emergency situation in the EPPO region linked to the outbreak of *Xylella fastidiosa*, EPPO revised Standard PM 7/24 in 2016 based on the experience gained in the region. With regard to the large number of host plants for this bacterium (359 plant species, EFSA 2016) and the complexity of *Xylella fastidiosa* infection (e.g. its uneven distribution within a plant and a potential absence of disease symptoms), a number of important knowledge gaps still need to be filled, such as field sampling, sample preparation with respect to different matrices, and diagnosis in insect vectors. Two Euphresco projects ('Harmonized protocol for monitoring and detection of *Xylella fastidiosa* in its host plants and its vectors' and '*Xylella fastidiosa* and its insect vectors Cicadella') were recently approved to be funded and will provide valuable knowledge to be used when revising the EPPO diagnostic protocol.

Improving communication

In the different EPPO countries, information may not always circulate effectively and reach the National Reference Laboratories and other laboratories involved in official diagnostic activities. However, both EPPO and Euphresco provide platforms for communicating up-to-date scientific and technical information within a large group of member countries. EPPO, as an intergovernmental organisation, has been very active in supporting the sharing of knowledge and expertise from different countries, through the activities of Diagnostic Panels, through the organisation of scientific and technical conferences and workshops, and through the establishment and maintenance of various databases (such as the EPPO Database on [Diagnostic Expertise](#)). By focussing on the coordination of plant health research, Euphresco facilitates communication among the various plant health stakeholders: research funders, National Plant Protection Organisations and research institutes, and brings them together to work on shared priorities. In Euphresco, organisations from more than 50 countries on five continents (Africa, the Americas, Asia, Europe and Oceania) collaborate on a voluntary basis to jointly address plant health challenges. Cooperation enables members to benefit from additional knowledge and skills not available in a given country or region, and more practically, to use infrastructures or carry out research on pests in areas where they already occur.

The European Reference Laboratories: enhancing cooperation in diagnostics in the EPPO region

The new EU Regulation (EU 2017/625) on official controls entered into force on 27 April 2017 and foresees the establishment of European Reference Laboratories (EURL) whose activities will enhance diagnostic capability and strengthen diagnostic activities in the European Union (EU, 2017). Since the late 1970s, the European Commission has gradually been establishing a network of Reference Laboratories covering various fields, such as animal health and animal welfare (e.g. the EURLs on foot and mouth disease, Newcastle disease or feed additives) and food safety (e.g. the EURLs on mycotoxins or pesticides in cereals). However, to date EURLs on plant pests have not been established. During this period, the plant health sector developed a number of activities to address its very specific needs. Expert groups are currently advising the European Commission on the best approach for the establishment of Plant Health EURLs. Research activities fall within the mandate of the EURLs but are rarely undertaken, mainly because other priorities absorb the limited funding available for the implementation of the EURLs' work programme. Research, and its coordination, is the main objective



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of Euphresco: research priorities are identified by NPPOs and research funders, and projects are funded to support their policy and provide evidence on specific questions. Collaboration with the European Commission could enable the specific needs of the EU, including those of the EURLs, to be addressed by transnational research consortia. EPPO and Euphresco are looking forward to cooperating with the EURLs as one measure to more efficiently address the everyday threats linked to regulated plant pests, and contribute to high quality, harmonised plant health diagnostics in the EPPO region.

TABLE 1/ List of the most recent Euphresco projects with a diagnostic objective (projects funded since 2015).

Title	Activity
The biology and epidemiology of ' <i>Candidatus Liberibacter solanacearum</i> ' and potato phytoplasmas and their contribution to risk management in potato and other crops	TD, TPS
Identification and early detection of <i>Cryphonectria parasitica</i> and <i>Ceratocystis platani</i> occurring on trees in Europe	TPS
VirusCollect II: building an international network of reference collections for regulated and other important plant viruses and viroids	RM
Assessment of <i>Dickeya</i> and <i>Pectobacterium</i> spp. on vegetables and ornamentals	TD
Consensus detection and identification protocol for <i>Acidovorax citrulli</i> on cucurbit seeds	TPS
Harmonized protocol for monitoring and detection of <i>Xylella fastidiosa</i> in its host plants and its vectors	TD, TPS, PT
Determine different <i>Plum pox virus</i> strains in wild hosts and in stone fruit cultivars with different susceptibility as a part of improved control and surveillance strategies	RM
Ringtest for improved <i>Potato virus Y</i> strain detection	PT
Tracking vectors of bacteria and phytoplasmas threatening Europe's major crops	TD
The application of Next-Generation Sequencing technology for the detection and diagnosis of non-culturable organisms: viruses and viroids	TD, TPS
Development, validation and verification of a diagnostic tool for detection and identification of <i>Ralstonia solanacearum</i> and <i>Clavibacter michiganensis</i> subspecies <i>sepedonicus</i> directly on plant tissue	TD, TPS, RM
Identification protocols for analysis of aquatic plants imports	TD
Inventory of living collections of cyst and root knot nematodes in Europe and their maintenance techniques	RM
Monitoring, detection and identification of the Japanese flower thrips <i>Thrips setosus</i>	TD
Understanding Little Cherry Viruses through improved diagnostics and insight in the occurrence and epidemiology	TPS, RM
Global warming and distribution of root-knot nematode species of the tropical group	TPS
Test performance studies on detection tests for ' <i>Candidatus Liberibacter solanacearum</i> '	TPS
Development of detection methods for viruses on potato (PVT, APMoV) and APLV/ APMoV test performance study	TPS
Study on the diversity of phytoplasmas detected in European forests	TPS
Assessment of a generic method for the detection of Begomoviruses	TPS
Improvement in diagnostic tests for quarantine pathogens by digital PCR	TPS, RM
Use of barcoding, from theory to practice	PT
Epitrix II	TPS
<i>Xylella fastidiosa</i> and its insect vectors <i>Cicadella</i>	TD, TPS
Comparison of real-time PCR detection methods for the plant pathogen ' <i>Candidatus Liberibacter</i> ' spp. causing Huanglongbing disease on <i>Citrus</i> spp.	TPS

TD: test development/optimisation; TPS: test performance study; PT: proficiency test; RM: reference material.

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More information on projects can be found [here](#).

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Summary of the EPPO Workshop on Flexible Scope

Wageningen, the Netherlands,
26 to 28 June 2017

Françoise Petter¹ & Madeleine McMullen¹

Background

Historically, the accreditation of laboratories was generally based on a fixed scope which should clearly and unambiguously define the tests covered by the laboratory's accreditation (e.g. immunofluorescence test for the detection of *Ralstonia solanacearum* on potato tubers). However, this does not readily allow new or modified tests to be added to a laboratory's scope, even when the competence of the laboratory in performing and validating related tests has already been evaluated by an accreditation body. Although applications for an extension to scope can be made at any time, the timescales involved may prevent rapid responses to client requests. Consequently, the concept of flexible scope has been developed. A flexible scope of accreditation allows a laboratory to undertake certain tests, and to report the results as accredited, even though these tests are not explicitly stated in the laboratory's scope.

Discussions between EA (European co-operation for Accreditation) and EPPO started in 2015 on the best way to implement flexible scope in plant pest diagnostic laboratories and to propose a regional harmonised approach. The main challenge faced by plant pest diagnostic laboratories when considering accreditation in the framework of official diagnostics is that there are over 300 pests (bacteria, fungi and chromista, insects and mites, nematodes, phytoplasmas, viruses and viroids, and invasive plants) recommended for regulation as quarantine pests by EPPO. Official plant pest diagnostic laboratories also perform analyses on exported plants and plant products for pests that are regulated by importing countries, in order to fulfil their international obligations under the International Plant Protection Convention (FAO, 1987). Laboratories in the EPPO region may then potentially need to test for hundreds of pests on thousands of pest/host matrices, under accreditation. The number of hosts affected by a pest may increase over time and, in particular, pests may increase their host range when they invade new areas and encounter potential new host plants. In addition, depending on their biology, they may be found in different parts of the plants or plant products, e.g. in the roots, leaves, fruits, and woody parts or packaging materials. Pests may also be present in substrates such as soil and water. For example, the stem nematode *Ditylenchus dipsaci sensu lato (s.l.)* attacks more than 1 200 species of wild and cultivated plants; it can attack

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aerial parts of plants but also bulbs, tubers and seeds and may be present in soil. As a consequence, the number of possible matrices to be tested and that would need to be validated for a single pest can be huge. In addition, different life stages of some of the pests (e.g. eggs, larvae, and adults for insects) may be present and require different tests to be used.

The Workshop

An EPPO Workshop on flexible scope was organised and held in Wageningen, the Netherlands from 26 to 28 June 2017 with the collaboration of the Dutch National Reference Centre of the NPPO. The Workshop followed the series of Workshops on Quality Assurance and Accreditation. It was attended by 46 participants from 24 countries. Participants included laboratory managers, quality managers, and representatives from accreditation bodies.

The aim of the workshop was to share experiences at the different laboratories concerning flexible scope in order to improve harmonisation of approaches in the EPPO region. It was considered that this harmonisation process was urgently needed because requirements for laboratories to be accredited for all their activities in plant pest diagnostics are increasing. The workshop started with a plenary session, including presentations by the laboratories and the EA of their experience with flexible scope. Three small workshops, organised in parallel, followed the plenary session to discuss practical issues on validation, quality assurance and expertise as part of the diagnostic process, in the framework of flexible scope.

The initial output expected from the workshop was to identify the main components that should be included in an EPPO Standard on flexible scope. However, during the workshop, it became clear that a more suitable approach would be to revise the two existing Standards on Quality Assurance: PM 7/84 *Basic requirements for quality management in plant pest diagnosis laboratories* and PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* (see below).

A separate meeting of a small drafting group composed of experts from the EPPO Panel on Diagnostics and Quality Assurance followed. Revisions of PM 7/84 and PM 7/98 were prepared by the drafting group and the revised standards will be sent for country consultation by the end of October 2017.

A Scientific Officer from EPPO attended the meeting of the Laboratory Committee of EA in September 2017 and presented the outcomes of the workshop, which were very much welcomed.

The presentations and some details on the outcomes are available at http://archives.eppo.int/MEETINGS/2017_conferences/Workshop_flexible_scope.htm



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EPPO Workshop on the Maintenance of Nematode Collections

Wageningen, the Netherlands,
5 to 6 September 2017

Madeleine McMullen¹ & Françoise Petter¹

The ability of National Plant Protection Organisations (NPPOs) to quickly and reliably detect and identify nematodes is critical for effective phytosanitary measures to be taken and to ensure safe transnational movement of plants and plant products. Laboratories are increasingly working under quality assurance systems (including accreditation) and need to have access to validated tests. In order to enable the development, validation and use of tests for the detection and identification of nematodes, it is essential that reference materials be well maintained and accessible. From 1 October 2013 to 30 September 2015, an EU project named Q-collect was funded by the EU Commission. The project aimed to improve the status of reference collections that are important to plant health, and the objectives of the project were to:

- make an inventory of existing plant pest collections in the European and Mediterranean region, and of their content,
- develop guidelines for quality standards,
- develop guidelines to improve the accessibility of these collections,
- design and build a network of reference collections,
- develop an info-portal on the internet to provide information on the outcomes of the project,
- disseminate results to stakeholders.

The project also aimed to support a network of national reference collections relevant to national and EU phytosanitary policy and to provide guidelines for preserving, maintaining and improving the quality and accessibility of national reference collections (specimens, tissue and DNA), thereby ensuring harmonisation of collection maintenance across Europe. The outcomes of the project were reviewed by the Panel on Diagnostics in Nematology, and the need for a specific workshop on nematode collections was identified to provide more guidance for nematology collections, in particular the maintenance of live collections. In addition, a Eupresco project on live collections of cysts and root knot nematodes had started and the Panel considered that a workshop would also provide greater opportunities to exchange information on such collections. The workshop was organised in conjunction with the Dutch National Reference Centre of the NPPO and was held on 5 and 6 September 2017 in Wageningen, the Netherlands. It was attended by 29 participants from 15 countries.

¹ EPPO, Paris, France



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The workshop started with a plenary session, with presentations from participants about the maintenance of their collections and on projects for collections.

The participants agreed that guidance on nematode collections should be drafted and that preparing an EPPO Standard would ensure that such guidance reached a large audience, as is the case with the EPPO Standard on Nematode Extraction. The main topics to be covered in this type of standard were identified, including the different types of collections (live/dead/*in silico*), their maintenance, culturing and storage, purification, and the metadata that should be available on biological material. Nineteen of the workshop participants agreed to send their Standard Operating Procedures on maintaining collections in their laboratories (translated into English or French) to the EPPO Secretariat by the end of 2017. As a next step, the SOPs will be used by an expert working group to draft an EPPO Standard on the maintenance of nematode reference materials.

More details on the meeting and presentations are available at

http://archives.eppo.int/MEETINGS/2017_conferences/Workshop_nematode_collections.htm





Contributing organisations for this issue:

ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail <i>French Agency for Food, Environmental and Occupational Health & Safety</i> www.anses.fr	Maisons-Alfort	France
AECOSAN	Agencia Española de Consumo, Seguridad Alimentaria y Nutrición / <i>Spanish Agency for Consumer Affairs, Food Safety and Nutrition</i> http://www.aecosan.msssi.gob.es	Madrid	Spain
AGES	Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH/ <i>Austrian Agency for Health and Food Safety Ltd</i> www.ages.at	Vienna	Austria
APHA	Animal and Plant Health Agency www.gov.uk/government/organisations/animal-and-plant-health-agency	Addlestone / Weybridge	United Kingdom
BfR	Bundesinstitut für Risikobewertung <i>Federal Institute for Risk Assessment</i> www.bfr.bund.de	Berlin	Germany
CODA-CERVA	Centrum voor Onderzoek in Diergeneeskunde en Agrochemie / <i>Centre d'Etude et de Recherches Vétérinaires et Agrochimiques</i> <i>Veterinary and Agrochemical Research Centre</i> www.coda-cerva.be	Brussels	Belgium
EVIRA	Elintarviketurvallisuusvirasto <i>Finnish Food Safety Authority</i> www.evira.fi	Helsinki	Finland
FERA Science Ltd	www.fera.co.uk	York	United Kingdom
FLI	Friedrich Loeffler Institut <i>Federal Research Institute for Animal Health</i> www.fli.de	Riems	Germany
ISS	Istituto superiore di Sanità <i>Italian National Institute of Health</i> www.iss.it	Rome	Italy
IZSAM	Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise <i>Experimental Zooprophyllactic Institute of Abruzzo and Molise</i> www.izs.it	Teramo	Italy
IZSLER	Istituto zooprofilattico della Lombardia e dell'Emilia Romagna <i>Experimental Zooprophyllactic Institute of Lombardy and Emilia Romagna</i> www.izsler.it	Brescia	Italy
NVWA	Nederlandse Voedsel en Warenautoriteit (Ministerie van Economische Zaken) / <i>Netherlands Food and Consumer Product Safety Authority (Ministry of Economic Affairs)</i> www.nvwa.nl	Wageningen / Utrecht	Netherlands
EPPO/OEPP	European and Mediterranean Plant Protection Organization <i>Organisation européenne et méditerranéenne pour la protection des plantes</i> www.eppo.int	Paris	France
PIWET (NVRI)	Państwowy Instytut Weterynaryjny, Państwowy Instytut Badawczy <i>National Veterinary Research Institute</i> www.piwet.pulawy.pl	Pulawy	Poland
SCL	Service commun des laboratoires <i>French Ministries for Economy and Finances</i> www.douane.gouv.fr / www.economie.gouv.fr/dgccrf/Le-service-commun-des-laboratoires-DGDDI-et-DGCCRF	Paris	France
SVA	Statens Veterinärmedicinska Anstalt / <i>National Veterinary Institute</i> www.sva.se	Uppsala	Sweden
WBVR	Wageningen Bioveterinary Research http://www.wur.nl/en/Expertise-Services/Research-Institutes/Bioveterinary-Research.htm	Lelystad	Netherlands

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