European Union Reference Laboratory for Bee Health

WORK PROGRAMME 2015

I. Legal duties

The functions and duties of the European Union Reference Laboratory (EU RL) for bee health are described in Commission Regulation (EU) No 415/2013 of 6 May 2013 laying down additional responsibilities and tasks for that laboratory and amending in Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council and repealing Regulation (EU) No 87/2011.

II. Objectives for 2015

Summary:

In 2015, the main activities of the EU RL should focus on:

- the animation of the National laboratory network on bee diseases through the organisation of the annual workshop, a ring test and a training on honeybee diseases;
- the statistical data analysis from the two years of EPILOBEE programme and the production of scientific publications;
- the support to the European commission, upon request, in the development and drafting of an epidemiosurveillance project;
- the documentation through a questionnaire of the analytical capacity available in each member state (MS) for pesticide analysis;
- the adaptation of GC-MS/MS methods (gas chromatography–tandem mass spectrometry) developed in previous years for the detection and quantification of several pesticides in apicultural matrices

Activity 1. Coordination of the network of NRLs for honeybee health

Description:

The coordination of the NRL network for honeybee health is an important task for the EU RL. In addition to the European NRLs, other NRLs from third countries are involved in this network: Kosovo, Norway, Switzerland and Kenya (African reference laboratory for honey bee health co-financed by the EC). The EU

RL is regularly contacted by new NRLs from third countries (e.g. in 2013-2014: Canada, Algeria and Jordania). The EU RL should animate the network by organising an annual workshop to inform the NRLs on the achievement of the work programme for the different activities and on the news related to honey bee health. This annual workshop is the opportunity to exchange information with the NRLs, to collect news from the partners (e.g.: necessity to produce new leaflets dedicated to a specific pathogen, requests for technical support, protocols, and/or reference materials - see Activity 3 on various honeybee diseases) and to harmonise diagnostic methods (a ring test and a training meeting are planned in 2015). The ring test will focus on *Nosema* typing (see section 3.2 Parasitology programme). The training will aim at harmonising the case definition for the identification of clinical signs from some honey bee diseases according to the needs expressed by the NRLs (e.g.: varroosis, European foulbrood, chronic paralysis).

Moreover, in 2015, the EU RL will submit a questionnaire to the network to gather information on the analytical capacities of the NRLs on pesticides.

In the framework of the network, the EU RL regularly receives insect samples from the European NRLs. It is worth noting that the EPILOBEE programme (see Activity 2. Epidemiology) was the opportunity to collect samples and to increase the surveillance of the introduction of exotic arthropods in Europe. These arthropods, previously identified or not by the NRLs, are very useful in all cases to feed the reference collections of the EU RL. In order to obtain relevant information on the samples for robust collections, the EU RL will produce a leaflet summarizing the requested information.

A collaborative website has been published on the internet in 2014. In 2015, the EU RL should feed the website with documents (leaflets, protocols, material reference requests, proficiency test reports) and with information useful for the NRL network. In addition to this website, the animation of the network should be continued through the dedicated email address.

Objectives:

- **Coordination of the NRL network.** It should rely on the animation of the network via the collaborative website, the organisation of the **annual workshop** and the dedicated email box.
- Scientific and technical support to the NRLs:
    - Information on surveyed pathogens and diseases
    - Advice on analytical methods, supply of protocols developed and used by the EU RL
    - Supply of biological material (positive and negative analytical controls)
    - Confirmatory diagnosis
- A questionnaire focused on the chemical analyses should be filled in by each NRL. It should record data on NRL analytical capacities in terms of material resources (devices available) and activities concerning the analysis of pesticides in bees and bee products. These results should help to follow and harmonise the activities of the network.
- **A summary of the questionnaire results** for 2014 should be produced by the end of 2015.
- A training focusing on the observation and the identification of clinical signs from some honeybee diseases according to the needs expressed by some NRLs (e.g.: varroosis, European foulbrood, chronic paralysis) will be organised.

In the light of the previous year experience some meetings between EU RL staff (head - Magali Chabert-Ribière, deputy head - Marie-Pierre Chauzat, the epidemiologist in charge of the epidemiological study) and the EC officers are needed. Consequently the cost of 2 travels for 2 persons to Brussels has been included in the 2015 budget.

Expected outputs:

- Organisation of the annual workshop
- Production of the annual workshop minutes
- Production of supporting documents and presentations for the workshop
- Organisation of a training on the identification of clinical signs from some honeybee diseases
- Production of supporting documents and presentations for the training
- Production of a questionnaire on the data from the NRL activities in the chemistry field
- Production of a report on the data from the NRL activities in 2013 (data collected in 2014)
- Production of a leaflet gathering information requested when sampling insects

Activity 2. Epidemiology

Description:

One of the priority missions of the EU RL for Honeybee health is to provide support to the EC with the finalisation of the epidemiological study on honeybee colony losses in Europe (EPILOBEE). This study involved a substantial number of MSs (17) and stretched from September 2012 to September 2014.

Apart from the surveillance of colony losses (overwintering colony mortality, season colony mortality), the major honeybee diseases were also surveyed during this study. The clinical prevalence of varroosis, American foulbrood (AFB), European foulbrood (EFB), nosemosis and chronic paralysis (CBPV) was estimated for the first year of the project (2012-2013) and should be estimated for the second year (2013-2014). The *Varroa destructor* infestation rate before winter should be statistically analysed.

The high number of MSs participating to the study, the large amount of data remaining to be processed and analysed and the need for day-to-day communication with the network, request appropriate staff allocation to the study which justifies the budget required for the staff effort. Data should be published through scientific papers co-authored by the consortium of participating countries.

As for the previous years, the EU RL will provide scientific support to the Commission, upon request, for the conception and development of epidemiological programmes.

It should be noted that a project on the sequencing of European *P. larvae* strains collected through EPILOBEE (cf. Activity 3.1 Bacteriology programme) should provide a substantial added-value to the scientific and technical efforts supplied in EPILOBEE programme.

Depending upon the necessity of the project and the requests of the EC, the objectives should be:

- Scientific and technical support to the EC:
    - Provide advices and guidance on the coordination of the epidemiological study EPILOBEE: evaluation of follow-up projects for each MS, processing the data and writing the scientific reports, presentation of the results to the MSs and to the EC.
    - Provide help on the implementation of epidemiological surveillance systems, including writing the guidelines and protocols, definition of sampling, drafting the questionnaires, collection and management of the data, start of the development of a database.

- Scientific and technical support to the MSs for the coordination of EPILOBEE
    - Advices and guidance on the data entry in the European database, management and processing of data
    - Technical support: models for the forms for the visit 2 and 3 in 2015, although specific co-fundings from the EC is not planned ; maintenance and improvement of the database
- Organisation of a training on EPILOBEE to exchange on practical aspects, data and valorisation of the results

Expected outputs:

- Submission of a paper to a scientific journal with impact factor on the EPILOBEE results for 2012-2013
- Production of a technical report on the data 2013-2014
- Production of the minutes for the training meeting
- Study of the feasibility of web-based interfaces to automatically calculate the epidemiological indicators

Activity 3. Work programme on the various honeybee diseases

Description:

In 2015, the EU RL work program will focus on the major honeybee diseases and pathogens:

- the two main bacteria responsible of the American and the European foulbroods (*Paenibacillus larvae, Melissococcus plutonius*, respectively*);*
- the main parasites present in Europe: the parasitic mites *Varroa destructor, Nosema* spp. and, to a lesser extend, *Acarapis woodi*
- the exotic parasites *Tropilaelaps* mites and *Aethina tumida* ;
- the five major viruses (CBPV, SBV, ABPV, DWV and BQCV).

The EU RL will provide assistance to the NRL network by supplying protocols and reference materials and should initiate the harmonisation of the analytical techniques by organising:

- A ring test on *Nosema* typing,
- A training on the identification of clinical signs from some honey bee diseases according to the needs expressed by some NRLs (e.g.: varroosis, European foulbrood, chronic paralysis).

Activity 3.1. Bacteriology programme

Description:

There is a wide range of methods currently used by the NRLs for diagnosing American foulbrood (AFB) and European foulbrood (EFB) and for detecting and identifying the primary causal agents *P. larvae* and *M. plutonius*. Among the set of methods already available, the EU RL validated a real-time PCR quantification assay for identification and quantification of *P. larvae* in 2014. A publication on the validation was also written in 2014 and will be finalized and submitted to a scientific journal in 2015. The validation of a similar real-time PCR quantification assay for *M. plutonius* was engaged in 2014 and should be pursued in 2015.

Objectives:

- **Publication of the real-time PCR quantification assays for the identification and quantification of *P. larvae* (AFB)**

The validation of the real-time PCR assays for identifying and quantifying *P. larvae* performed in 2014 has been written. It should be finalized and submitted to a scientific journal for publication in 2015. This step of publication will officially produce a validated method for the NRLs and the international scientific community.

- **Validation of the real-time PCR quantification assays for the identification and quantification of *M. plutonius* (EFB)**

The validation of the real-time PCR assays developed in 2014, for identifying and quantifying *M. plutonius* should be pursued. The method should be validated in accordance with the criteria of the French Standard XPNF U47-600 for animal health analysis methods - PCR.

- **Assessment of the feasibility to improve the molecular identification of *P. larvae***

In 2014, a ring test on *P. larvae* identification by PCR analysis was organised by the EU RL. The results of the ring test showed that, among the 26 participating laboratories, 21 were satisfactory. Three of the five laboratories that returned unsatisfactory results used a PCR protocol described in the OIE Terrestrial Manual. Moreover, three laboratories, assessed as satisfactory and that used an OIE method, informed the EU RL that they encountered various problems. In view of this information, the EU RL will assess the possibility to improve the identification of *P. larvae* by conventional PCR analyses. Some experiments will be dedicated to this issue.

- **Sequencing the European strains of *P. larvae***

In the framework of the EPILOBEE programme, samples of brood diseased of AFB were collected. The EURL will conduct a project on whole genome sequencing of European *P. larvae* strains. The project would be the first high-throughput sequencing project at the European scale. It would allow to identify conserve genome regions of *P. larvae* that could be potential targets for future diagnosis tools. Indeed, several tools are available for *P. larvae* detection and/or identification, but some of them turned out to be non-specific. This was revealed through the comparative laboratory test that the EU RL conducted in 2014, about the identification of *P. larvae* in DNA samples by PCR analyses. The samples targeted for this

P. larvae high-throughput sequencing project are already available in the participating countries and the project should only induce little additional work (preparation of the samples, packing and shipping). The EU RL will ensure the collection, selection and preparation of the samples and the final analysis of the results. The sequencing step will be performed by the Anses Genomic Platform. These costs have been included in the budget.

- **Internal stock-culture collection**

The EU RL maintains and updates an internal stock-culture collection with bacterial isolates and strains from different geographical origins to have an epidemiological overview of the bacterial landscape in Europe. NRLs should be requested to contribute to the supply of the collection.

Expected outputs:

- Publication of a paper on the real-time PCR method for the detection and quantification of *P. larvae* in a scientific journal
- Finalisation of the writing up for the validation of the real-time PCR method for *M. plutonius*
- Production of an updated reference list with strains and isolates available in the EU RL stock culture collection (number of strains and isolates in the stock culture collection)
- Number of reference materials and protocols provided to the NRLs
- Collection of *P. larvae* strains coming from EPILOBEE programme and data from sequencing

Activity 3.2. Parasitology programme

Description:

Several methods are currently used by the NRLs for identifying *Nosema apis* and *Nosema ceranae*. A ring test should be organized to compare and to evaluate the sensitivity and specificity of PCR methods used by the NRLs in the purpose of evaluating the level of harmonisation of the diagnostic techniques used throughout the European Community.

The tracheal mite *Acarapis woodi* is still present in European colonies although the prevalence of the disease had decreased due to acaricide treatments used to control varroa mites. However, it is important to better detect the mite molecularly and to better identify individuals morphologically. These methods of diagnosis should allow differential diagnostics from other mite-related diseases.

Objectives:

Nosemosis:

- **Comparative laboratory test/ Ring test on molecular identification of *Nosema* species**

All the NRLs should be invited to participate to a ring test for the identification of *N. apis* and *N. ceranae* with molecular techniques. The specificity of the molecular techniques used by the NRLs should be tested on a panel of samples of *N. apis* and *N. ceranae* and of other pathogens living in the hive. These results should allow estimating the need for harmonisation in Europe for these molecular methods.

- **Internal stock-culture collection**

The EU RL maintains an internal stock-culture collection with *Nosema* species isolates from different geographical origins. The collection includes the two species of *Nosema* pathogen to honeybees and also

other genetically similar microsporidies (e. g. *N. bombi*). The team should continue to update this collection as it is a key tool to validate the diagnostic methods.

Acariosis:

- **Internal stock-culture collection**

The EU RL maintains and updates an internal stock-culture collection with *Acarapis woodi* isolates from different geographical origins. The collection should also include other genetically similar *Acarapis spp.*, difficult to obtain for various reasons (some species are present in Asia only, species are difficult to be identified). During 2015 the EURL team will boost collaboration with colleagues from Asia in the purpose to obtain these species.

- **Tests of molecular identification methods**

Acariosis is caused by the tracheal mite *A. woodi* which parasitises the respiratory system, living and reproducing mainly in the large pro-thoracic trachea of the honeybees. The detection of these very small mite performed by dissection of the adult honeybee trachea is highly time consuming.

Among the set of methods described in the literature, the EU RL tested in 2014 a conventional PCR assay based on the published work of Kojima, *et al*. (2011)[1]. In 2015, the tests of this method to detect and identify *A. woodi* should be pursued. The progress of this work is related to the availability of biological material.

Expected outputs:

- Production of a report from the comparative laboratory test on the capacity of the NRLs to identify *Nosema* species by molecular-based methods
- Production of an updated reference list with isolates available in the stock culture collection (number of strains and isolates in the stock culture collection) for *Nosema spp*. and *Acarapis spp*.
- List and number of reference materials (plasmid constructions) and protocols provided to the NRLs

Activity 3.3. Entomology and exotic arthropod programme

Description:

The work programme for 2015 plans to continue working on molecular identification methods for the exotic diseases *Aethina tumida* and *Tropilaelaps* spp.. A collection of reference materials (adults, larvae and eggs) has been initiated in 2012 for both parasites. In the purpose of discrimination, the collection also integrates genetically similar parasites and parasites that can be found in honeybee colonies. The 2015 EU RL programme plans to pursue enlarging the collection.

[1] KOJIMA Y., YOSHIYAMA M., KIMURA K. and KADOWAKI T. (2011) PCR-based detection of a tracheal mite of the honey bee *Acarapis woodi*, *Journal of Invertebrate Pathology* **108,** 135–137

Objectives:

Aethina tumida

- **Finalisation of the validation of the real-time PCR method for *A. tumida* identification**

The validation of the real-time PCR assays developed in 2014 for identifying *A. tumida* should be pursued. The real-time PCR assay performances should be assessed by determining the specific characteristics, such as analytical specificity, limit of detection (LOD_{PCR}) and limit of the method. The assays should be performed in accordance with the criteria of the French Standard XPNF U47-600 for animal health analysis methods - PCR. The results should allow the validation of the method.

- **Internal stock-culture collection**

The EU RL internal stock-culture collection started at the beginning of the EU RL mandate is supplied with new materials every year. Since the EU RL has detected a failing in the sensitivity of a PCR method previously used, the collection has to be enlarged with coleopteran specimens from various geographical origins. In 2015, the collection should be enlarged with *A. tumida* specimens originated from various parts of the world.

***Tropilaelaps* spp.**

- **Morphological identification of *Tropilaelaps* spp.**

In 2014, a training was organised by the EU RL on the morphological identification of the two exotic arthropods, *Tropilaelaps* spp. and the small hive beetle. In parallel to the training the EU RL submitted to accreditation a method for the morphological identification of *Tropilaelaps* spp. This method should be published and disseminate in 2015.

- **Improvement of the PCR method for the identification of *Tropilaelaps* species**

A conventional PCR method, associated with a sequencing step, has been developed in the laboratory for a better identification of *Tropilaelaps* species. This method, adapted from a published study [2], allows the identification of *Tropilaelaps* specific species. The specificity of the method has been improved by testing a panel of various *Tropilaelaps* spp. and mites frequently observed in the hive. In 2015, tests on the specificity of the method should be continued. The writing up of a scientific publication should be started.

- **Development of a real-time PCR method for the identification of *Tropilaelaps* species**

In order to overcome the sequencing step, the EU RL should develop an internal method for the identification of the four species of *Tropilaelaps* (*T. clareae*, *T. koenigerum*, *T. mercedesae* and *T. thaii*) based on the HRM (High-Resolution Melt) analysis. HRM is a real-time PCR-based method to analyse DNA melt curves which allows the detection of mutations between similar sequences. In 2014, a portion of the COI gene has been investigated and plasmid constructions are available as reference materials. In 2015, the development and the validation of this internal method should be continued.

[2] ANDERSON D.L. and MORGAN M.J. (2007). Genetic and morphological variation of bee-parasitic *Tropilaelaps* mites (Acari: Laelapidae): new and re-defined species. *Exp. Appl. Acarol.*, **43**, 1–24.

- **Internal stock-culture collection**

The EU RL internal stock-culture collection started at the beginning of the EU RL mandate is supplied with new materials every year. In 2015, the collection will be enlarged with *Tropilaelaps* specimens, from laboratories specialised in honeybee mites and located in various continents. The objective is to obtain specimens of the four species of *Tropilaelaps* originated from various countries and specimens of acarian closed to *Tropilaelaps* to validate the methods and provide materials for the NRLs network.

Expected outputs:

- Production of the validation steps for the real-time PCR method for *A. tumida* identification
- Finalisation of the validation documents for the *A. tumida* molecular identification and preparation of a scientific publication
- Production of the first steps for the *Tropilaelaps* species identification through HRM analyses
- Production of an updated reference list with *Tropilaelaps* and *A. tumida* specimens available in the stock-culture collection (number of specimens in the collection and origin)

List and number of protocols provided to the NRLs

Activity 3.4. Virology programme

Description:

The work programme for 2015 plans to finalize with the ongoing work on the development and validation of reference methods for the detection and the quantification of the five major honeybee viruses that threaten the health of the colonies: the chronic bee paralysis virus (CBPV), the sacbrood virus (SBV), the black queen cell virus (BQCV) and the two viruses acting in synergy with the mite *Varroa destructor,* the acute bee paralysis virus (ABPV) and the deformed wing virus (DWV). Moreover, the EU RL should initiate a collaborative work with some NRLs in order to evaluate the accuracy of the CBPV threshold (virus quantity per bee) that is used in Europe for the diagnosis of the chronic paralysis disease.

Objectives:

- **Validation of real-time PCR quantification assays for BQCV**

The real-time PCR method for BQCV quantification in bees should be finalized including the detection limit and the specificity (exclusivity). The real time PCR developed in 2014 revealed a wild repartition of this virus, as all the tested bees were positive. A hive screening should be implemented in order to find non-infected bees. Subsequently, the validation process should be continued. The results should be submitted for publication.

- **Characterisation and validation of a quantitative PCR for ABPV and DWV**

As performed in 2014 for the ABPV, the EU RL should implement the first experimental steps of real-time PCR for the detection and quantification of the DWV in accordance with the AFNOR Standard NF U47-600 for the validation. In 2015, the validation documents should be produced for ABPV and DWV.

- **Biological materials and inter-laboratory test on CBPV quantification**

The EU RL plans to organize an inter-laboratory test on CBPV quantification during the winter 2014/2015. The inter-laboratory reproducibility characteristics of the CBPV quantitative PCR method should be

evaluated. Positive samples of CBPV produced in 2014 completed with negative bees collected at fall 2014 should be sent to the participants (NRLs) for this inter-laboratory test. Participants should receive and blind test the samples in early 2015. The validation document should be finalised and should include the inter-laboratory reproducibility characteristics.

NRLs, experts and reference laboratories from third countries should continue their contribution to supply the viral stock collection for the purpose of method validation.

- **Threshold for chronic bee paralysis diagnosis**

During the EPILOBEE meeting 2014, the question of the accuracy of the threshold used to define a case of chronic bee paralysis (10^8 CBPV RNA copies per bee) was raised. This threshold was defined using honeybees located in France. The EU RL should initiate the collection of information from the NRLs on CBPV loads measured in symptomatic and non-symptomatic bees in the other MS. The accuracy of the threshold should be evaluated and eventually modify according to this new data.

Expected outputs:

- Submission of the method for the quantification of BQCV to a scientific journal
- Production of the document for the validation of the method for ABPV and DWV quantification
- Production of a report on the inter-laboratory test on the CBPV quantification method

Activity 4. Pesticide programme

Description:

Continuing the work begun by the EU RL in the previous years, the validation of methods for chemical screening in hive matrices will be completed. The 2015 work programme should focus on the validation and the publication of the methods developed during the previous years. To complete the set of methods already used by the EU RL for honeybee health, the laboratory should validate the multi-residue method on the beebread matrix during the year 2015. The multi-residue methods (organochlorines, organophosphorus and synthetic pyrethroid families) have been validated in honeybees and pollen by GC-ECD and GC-NPD, and should be submitted for publication in a scientific journal. The unambiguous identification and the confirmation of the presence of analytes in bees, pollen and beebread are necessary. If the initial analysis does not provide unambiguous identification or does not meet the requirements for quantitative analysis, a confirmatory analysis is required [SANCO/12571/2013].. Mass spectrometry coupled to a chromatographic separation method is a very powerful combination for the identification of an analyte in the sample extract. Therefore, a GC-MS/MS device is necessary in the laboratory to finalize the identification of the pesticides. Consequently the methods should be optimised and transferred on this instrument.
Concerning the neonicotinoid insecticides, the EU RL has already developed and validated the detection of imidacloprid, clothianidin, acetamiprid, thiacloprid and thiamethoxam residues in honeybees, beebread, pollen and nectar. The validation of the method developed in honeybee larvae is currently implemented and should be submitted for publication in a scientific journal in 2015.

Objectives:

- **Publication of the multi-residue method validated in honeybees and in pollen**

During 2014, the EU RL has finalised the validation of the multi-residue method for measuring organochlorines, organophosphorus and synthetic pyrethroid residues in pollen. In 2015, these methods (determination of pesticides in honeybees and in pollen) should be submitted to a scientific journal for publication.

- **Validation of a multi-residue method in beebread**

The multi-residue method for detection of more than 20 compounds from the organochlorines, organophosphorus and synthetic pyrethroid residues should be validated on the beebread matrix by GC-ECD and GC-NPD.

- **Preparation of a publication of the determination of neonicotinoid insecticide residues in honeybee larvae**

In 2015, the EU RL will finalise the production of the validation documents for the method for the detection and quantification of neonicotinoid insecticides in honeybee larvae. The writing up of a scientific publication should be started.

- **Optimization of the multi-residue methods developed in honeybees, pollen and beebread by GC-MS/MS**

The multi-residue method developed in honeybees, pollen and beebread should be adapted and optimised on GC-MS/MS as confirmatory method to finalize the identification of the pesticides. The cost of this material has been included in the budget.

Expected outputs:

- Submission of the multi-residue method validated in honeybees and in pollen to a scientific journal
- Validation of the multi-residue method for the detection and quantification of pesticides residues in beebread
- Preparation for publication of the method of the detection and quantification of neonicotinoid insecticides in honeybee larvae
- Adaptation and optimisation of the multi-residue method in honeybees, pollen and beebread on GC-MS/MS as a confirmatory method. Implementation of the first steps for the production of the validation document.