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EUROPEAN UNION REFERENCE LABORATORY (EU-RL) FOR BOVINE TUBERCULOSIS

WORK PROGRAMME 2015 – Version 1

VISAVET

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The purpose of the work programme is to cover the objectives (general, specific and operational) defined in the Annex to the Commission Implementing Decision establishing the work programme for the year 2015 on financial contribution to the European Union reference laboratories, taking into account the responsibilities and tasks defined in the Annex II to Commission Regulation (EC) No 415/2013 regarding the EU-RL for Bovine Tuberculosis:

1. To coordinate, in consultation with the Commission, the methods employed in the Member States for diagnosing bovine tuberculosis.

2. To facilitate the harmonization of techniques throughout the Union, in particular specifying standard test methodologies.

3. To organize workshops for the benefit of national reference laboratories as agreed in the work programme and estimated budget referred to in Article 2 of Implementing Regulation (EU) No 926/2011, including training of experts from the Member States and, as appropriate, from third countries, in new analytical methodologies.

4. To provide technical assistance to the Commission and, upon its request, to participate in international for a relating to the diagnostic of bovine tuberculosis, concerning in particular the standardization of analytical methods and their implementation.

5. To perform research activities and, whenever possible, coordinate research activities directed towards the improved control and eradication of bovine tuberculosis.

A. MAIN ACTIVITIES OF THE EU-RL FOR BOVINE TUBERCULOSIS FOR 2015.

1. Characterization of bovine PPDs used for the detection of immune response.

Operational objective 1: To ensure the development and use of high quality analytical methods across the EU-RL network.

Description: The *in vivo* diagnosis of bovine tuberculosis is based on the skin test that exploits bovine PPD as a reactive. PPD is a protein purified derivative produced by the precipitation of the soluble constituents (mainly proteins) of *Mycobacterium bovis* cultures. The procedure although simple and efficient leads to a product with variable composition within batches which makes difficult the standardization of the production and renders obligatory the comparison of each batch of the product with an international standard for the determination of its biological activity (potency).

Objectives: The main objective of this task will be to analyse the composition of bovine PPD in order to increment its performance and allow a better standardization of the reagent. More specifically, the EU-RL will intend to a) identify and quantify the main components of bovine PPD, b) study the biological activity of the various constituents of bovine PPD, and c) determine the components that can lead to cross-reactions and thus decrease the specificity of the assay. For this purpose, the EU-RL will apply state of the art techniques including production of monoclonal antibodies, MALDI TOF MS, chromatographic techniques and biological (*in vitro* and *in vivo*) assays.

Expected outputs: Understand the composition, detect the biologically active components and recognize the cross reacting constituents of bovine PPD. These three steps will permit in the future the production of PPDs with improved performance in terms of sensitivity and specificity and will allow the better standardization of the production process of this reagent.

2. Potency test of bovine PPDs.

Operational objective 1: To ensure the development and use of high quality analytical methods across the EU-RL network.

Description: The Purified Protein Derivatives (PPD or tuberculin) are the reagents for the official *in vivo* and *in vitro* diagnostic assays based on cell-mediated immune response for the diagnosis of bovine tuberculosis. The biological potency of a PPD is estimated by comparing the size of the reaction elicited by an intradermal inoculation of the test tuberculin and an International Standard in naturally infected cattle or experimentally infected or sensitized guinea pigs as defined in the European Pharmacopoeia and the OIE Manual.

Objectives: A comparative test is programmed at the end of 2014 including the NRLs that perform biological testing. Depending on the results, studies towards standardization of the protocol will be performed with the final objective of setting up a standardized protocol for performing potency test of tuberculins in guinea pigs. Moreover, tuberculins used in the Member States for bovine diagnosis will be tested to determine their biological potency.

Expected outputs: a) Standard protocol to determine the biological potency of the tuberculin in guinea pigs; b) Define the potency of the tuberculins supplied by different producers all over Europe.

3. Molecular detection of *Mycobacterium tuberculosis* complex in animal tissues.

Operational objective 1: To ensure the development and use of high quality analytical methods across the EU-RL network.

Description: During 2014 the EU-RL has developed an extraction protocol combined to a Real Time PCR assay for the detection of members of the *Mycobacterium tuberculosis* complex (MTBC) in animal tissues. The assay demonstrated remarkable sensitivity and specificity when compared to culture. For 2015 the EU-RL is seeking to further evaluate the performance of this protocol and finally validate it by performing studies on the specificity, repeatability and reproducibility of the assay.

Objectives: Main objectives of this task will be a) the definition of the sensitivity of the test by comparing it with skin test, pathology and culture, b) the estimation of the specificity by applying it on tuberculosis free herds, c) the evaluation of the repeatability by intralaboratory assays, and d) the estimation of the reproducibility in cooperation with NRLs that already apply molecular techniques for the detection of members of MTBC in animal tissues.

Expected outputs: The creation of a robust and efficient molecular protocol for the detection of members of the MTBC in animal tissues that once thoroughly validated could be exploited in the diagnosis of bovine tuberculosis.

4. Update in Mycobacteria recovery from different culture media.

Operational objective 1: To ensure the development and use of high quality analytical methods across the EU-RL network.

Description: In 2013 the EU-RL performed a study to define the sensitivity of different culture media to be able to recommend the media that should be used for isolation of Mycobacteria in the Member States. The conclusion of this study was to use a

combination of culture media (Lowënstein Jensen with pyruvate or glycerol, MGIT, Coletsos, Middlebrook 7H11) for the rapid detection of positive samples with the vigotous growth. The drawbacks of this study were that only four *Mycobacterium* spp. were included and all of them were from the same origin (Spanish isolates).

Objectives: The objective of this study will be to collect a representative number of isolates from the Member States (*M. bovis* and *M. caprae*) and to subculture them in the selection of culture media identified in the previous study. In this sense, it will be possible to identify the culture media that shows the best results regarding isolation of *M. bovis* and *M. caprae*.

Expected outputs: Recommended culture media for microbiological protocol within Europe.

5. Comparative tests.

Operational objective 1 and 2: To ensure the development and use of high quality analytical methods across the EU-RL network; and to mantain appropriate level of proficiency testting ensuring efficiency of control analysis methods.

Description: During 2015, two ring trials will be organized regarding microbiological culture and potency testing of PPDs. The rationales for organizing these ring trials are: a) bacteriological culture is defined as the diagnostic tool for confirming the disease and it is still considered as the gold standard. In this ring trial, the NRLs will adapt their culture media selection in accordance with the results obtained in the EU-RL activity "Mycobacteria recovery from different culture media"; and b) the EU-RL is performing studies in guinea pigs to determine the biological potency of the tuberculins used in the different Member States for the diagnosis of bovine tuberculosis. Although guidelines for the protocol are described in the OIE manual, some modifications have been encountered between NRLs. Although a comparative test is going to be organized in 2014, only a few PPDs can be tested in each ring trial and more information regarding protocols as well as performance of NRLs is needed. Participation of producers will be considered taking into account the results obtained in the first ring trial.

Objectives: To organize two ring trials for all the NRLs: b) Evaluation of the isolation of mycobacteria by microbiological culture. A set of tissue samples will be sent to NRLs to perform the bacteriological culture to define the positive and negative samples; b) Evaluation of the methodology for determination of biological potency of the PPD in guinea pigs. Blinded PPDs will be sent to determine the biological potency in each NRL.

Expected outputs: a) Evaluation of the methodology for bacteriological culture; b) Knowledge of the methodology for determination of the biological potency of tuberculins in guinea pigs and comparison of results.

6. Open databases.

Operational objective 1 and 3: To ensure the development and use of high quality analytical methods across the EU-RL network; and to ensure the availability of scientific and technical assistance provided by the EU-RLs.

6.1. MALDI BIOTYPER for the identification of mycobacterial species.

Description: MALDI TOF MS is proposed as an extremely promising approach for the identification of bacterial isolates based on their proteomic profile. This technique reduces considerably the time and resources needed for identification thus, rendering it ideal for the identification of mycobacterial species. The main drawbacks regarding mycobacterial identification by MALDI BIOTYPER today are the absence of an effective protocol for the extraction of high quality proteins from mycobacteria and the relatively small number of entries (mainly of human interest) in the commercially available platforms.

Objectives: a) Further elaboration and validation of the extraction protocol that the EU-RL has developed during 2014; and b) creation of a database, which will include several entries of the main mycobacterial species of veterinary interest.

Expected outputs: a) Production of a protein extraction protocol for the identification of mycobacterial species by MALDI TOF MS. This protocol once validated could be proposed as a reference standard protocol for the detection of mycobacteria by MALDI TOF MS; and b) creation of new mycobacterial entries of veterinary interest for the BIOTYPER database which will permit a more efficient and precise identification of mycobacterial species. The above-mentioned database could be the base for the creation of a European database with spectra of mycobacterial isolates, available to all Member States.

6.2. Molecular database.

Description: Nowadays, DVR-spoligotyping is still considered as the routine molecular characterization protocol for members of the MTBC. Two main databases are available: the SITVIT Database (Public Health, Demay et al. 2012) and the mbovis.org (Animal Health, Smith et al. 2012). Unification of both databases (*M. bovis*/*M. caprae*) will assist the epidemiological studies of bovine tuberculosis.

Objectives: To migrate the mbovis.org database to the VISAVET server (EU-RL website). This objective is the first step towards the final task of integrating both databases including a specific European database (ie. mbovisEU.org) that will be scheduled in the future activities for the EU-RL.

Expected outputs: Maintenance of a molecular database to guarantee the standardization of nomenclature for DVR-spoligotyping profiles allowing epidemiological studies.

7. European Standard.

Operational objective 1: To ensure the development and use of high quality analytical methods across the EU-RL network.

Description: The international standard (IS) (NIBSC, United Kingdom) is used as a control in the *in vivo* testing of the tuberculins although its stock is limited and questions regarding its actual quality are arising. For this reason, the EU-RL has been focusing in studying the characteristics needed for a future European Standard for *in vivo* testing of the tuberculins.

Objectives: The main objective would be the production of a European Standard tuberculin to be distributed to the stakeholders for their potency testing studies to avoid consuming the IS stock. During 2015, potency tests in guinea pigs will be carried out to test the potency and define the suitability of the European Standard to perform the potency studies. Moreover, the European Standard will be included in the studies programmed within section 1 (Characterization of bovine PPDs) as well as the ring trial.

Expected outputs: To test the European Standard *in vivo* (guinea pigs) and *in vitro* (analyse its composition) to guarantee its suitability as an internal control in the potency test studies.

8. Missions.

Operational objective 1, 2 and 3: To ensure the development and use of high quality analytical methods across the EU-RL network; to mantain appropriate level of proficiency testting ensuring efficiency of control analysis methods; and to ensure the availability of scientific and technical assistance provided by the EU-RLs.

Description: If requested by the EC or under specific circumstances, the EU-RL staff will visit the European Commission or NRLs. During 2015, a visit to the French NRL (OIE Reference Laboratory for Bovine Tuberculosis) is scheduled to organize the direct extraction protocol validation and to discuss the *in vivo* PPDs testing. Moreover, the EU-RL staff visits: a) cattle farms to perform field studies (MID-test, IFN-γ, serology) and

collect samples (blood) for the EU-RL samples bank; b) slaughterhouses to collect tissue samples (lymph nodes and organs) to perform the bacteriological culture and/or to be included in the sample reference bank; and c) CVO offices to discuss sampling of cattle farms. One objective of the EU-RL is to keep abreast of developments mainly in diagnosis and epidemiology of tuberculosis and therefore the EU-RL staff attend congresses, workshops, training courses and they are updated through reports from experts, legislation, scientific papers, etc.

Objectives: a) To assist the EC/NRLs; b) To collect biological samples (farm/slaughterhouse); and c) To attend conferences and training courses (ie. Introduction to Next Generation Sequencing, UK).

Expected outputs: a) Technical advice/collaboration with EC/NRLs; b) Collection of samples for field studies included in the WP; and c) Scientific training of the EU-RL staff.

9. Training of personnel

Operational objective 1, 2 and 3: To ensure the development and use of high quality analytical methods across the EU-RL network; to mantain appropriate level of proficiency testting ensuring efficiency of control analysis methods; and to ensure the availability of scientific and technical assistance provided by the EU-RLs.

Description: As included in the Annex II of the Commission Regulation (EC) No 415/2013, the EU-RL must train experts from the Member States. These training mobilities are designed to teach new methodologies as well as accreditation process and the workflow in a BSL 3.

Objectives: Short visits for two National Reference Laboratories to allow the establishment of new protocols and techniques in their laboratory of origin. The trainee will present the activities of his/her NRL to the EU-RL and will submit a brief report after the visit.

Expected outputs: Training of NRL staff in mycobacteria protocols (culture, PCR, DVR-spoligotyping, MIRU-VNTR, IFN-γ test) and accreditation system.

10. Workshop.

Operational objective 1, 2 and 3: To ensure the development and use of high quality analytical methods across the EU-RL network; to mantain appropriate level of proficiency testting ensuring efficiency of control analysis methods; and to ensure the availability of scientific and technical assistance provided by the EU-RLs.

Description: A workshop is an annual meeting for information and coordination for all National Reference Laboratories.

Objectives: To organize a workshop with all the NRLs to present the information regarding the activities performed by the EU-RL in the previous years and to understand the priorities and necessities regarding bovine tuberculosis diagnosis of the different NRLs.

Expected outputs: Annual meeting to share information regarding EU-RL activities with the NRLs.

B. OTHER ACTIVITIES OF THE EU-RL FOR BOVINE TUBERCULOSIS FOR 2015.

Operational objective 1, 2, 3 and 4: To ensure the development and use of high quality analytical methods across the EU-RL network; to mantain appropriate level of proficiency testting ensuring efficiency of control analysis methods; to ensure the availability of scientific and technical assistance provided by the EU-RLs; and to ensure a sound and efficient management of EU-RL funding clycle.

The following tasks will remain permanent activities of the EU-RL for 2015.

1. Preparation, control and supply of laboratory material (including in house spoligotyping membranes) and protocols.

2. Collection of representative samples (tissue samples, strains, DNA, serum/plasma).

3. Isolation, identification and typing of mycobacteria.

4. World Wide Web page update.

5. Technical assistance to the Commission and NRLs and participation with EFSA and international organizations (bovine tuberculosis subgroup of the Task Force, OIE).

6. Dissemination (presentations at international and national congresses or conferences, and publication in international and national journals).

7. Keeping abreast of developments (papers, conferences, training courses, reports, legislation, etc.) and research activities (collaboration with NRLs, participation in research projects, etc.).

8. Technical and financial management of the activities included in the work programme.