EUROPEAN COMMISSION

HEALTH & CONSUMERS

DIRECTORATE-GENERAL

Directorate D – Animal Health and Welfare

Unit D1- Animal health and Standing Committees

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

WORK PROGRAMME 2014 – PROPOSAL

VISAVET

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The objective of the work programme is to cover the responsibilities and tasks defined in the Annex II to Commission Regulation (EC) No 415/2013:

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 2014.

1. Assessment of diagnostic tests based in the immune response.

1.1. Cellular response.

Description: The IFN-γ test is used for detection of the IFN-γ against *M. bovis* and *M. avium* antigens. This technique is used together with the skin test to detect a higher number of infected animals. During 2012, a Scientific Opinion on the use of gamma interferon test for the diagnosis of bovine tuberculosis [EFSA Journal 2012;10(12):2975] has been published including some recommendations regarding harmonization of the protocol as well as the study of factors that may affect the test specificity. In the case this technique is incorporated to the EU legislation as well as the OIE Manual, there is a necessity to address the recommendations included in this opinion to have a robust, safe and reliable accepted protocol by all the MSs NRLs.

Objectives: To address the recommendations included in the EFSA Scientific Opinion on the use of gamma interferon test for the diagnosis of bovine tuberculosis (harmonised protocol, influence of factors-age, production system, etc.).

Expected outputs: a) To define the performance of the IFN-γ test when evaluating different factors under different epidemiological situations; b) To set up a harmonised protocol for IFN-γ detection in the EU.

1.2. Humoral response.

Description: Serology is not an official test for bovine tuberculosis although nowadays few tests are commercially available. Since the main objective in an eradication campaign is the detection of the highest number of infected animals, research studies combining the available diagnostic techniques (skin test, IFN-γ, serology, culture) should be performed in order to determine which combination is the most sensitive.

Objectives: To perform a preliminary study to determine the sensitivity of serology for the diagnosis of bovine tuberculosis. For this study a number of animals will be tested with the skin test, IFN-γ and serology and the disease will be confirmed by bacteriological culture.

Expected outputs: To define the performance of serology tests together with the official tests for diagnosis of bovine tuberculosis (skin test and IFN-γ).

2. Potency test of tuberculins.

Description: The Purified Protein Derivatives (PPD or tuberculin) are essential reagents for *in vivo* and *in vitro* diagnostic assays based on cell-mediated immune response. Regarding diagnosis of tuberculosis, the keystone in all the eradication programs all over Europe is the intradermal tuberculin test (single or comparative) and therefore the determination of the biological potency of the PPDs is crucial to be able to detect the maximum number of reactors. This potency is estimated by comparing the size of the reaction elicited by an intradermal inoculation and comparison the size of the reactions of a "standard" tuberculin of known potency (International Standard) in naturally infected cattle or experimentally infected or sensitized guinea pigs.

Objectives: Determine the potency test of tuberculins in guinea pigs (infected *vs.* sensitized) to define the standard protocol, based on the one described in the OIE Manual, that offers the most accurate result. Tuberculin sent by NRLs will be tested as well as the European and International standards. This activity will incorporate the results derived from the comparative test.

Expected outputs: a) Reduce possible deviations in the standard protocol to determine the biological potency of the tuberculin in guinea pigs; b) Define the potency of the tuberculins supplied by different manufacturers all over Europe.

3. Set up of a direct extraction technique from tissue samples.

Description: Although the bacteriological culture is defined as the reference technique to confirm the infection (Council Directive 64/432/EEC) efforts are being made to develop an alternative/complementary technique that is able to detect at least the same number of animals in less time. During the last years the EU-RL has been working in defining a protocol although preliminary results throw lower sensitivity (2013 results still needed to be evaluated) and the obtained DNA has not the right quality/quantity to perform molecular characterization techniques (DVR-spoligotyping and MIRU/VNTR).

Objectives: To design a protocol for direct extraction from tissue samples that allows the molecular characterization of the isolates belonging to the *M. tuberculosis* complex. A set of samples from skin test reactors will be tested by bacteriological culture and DNA extraction to compare the results regarding confirmation of infection and molecular characterization of the isolates.

Expected outputs: To define the best DNA extraction technique that allows the identification and characterization of the *M. tuberculosis* complex strains.

4. Comparative tests

Description: During 2013, two ring trials will be organized regarding potency testing of tuberculin and molecular characterization of strains. The rationales for organizing these ring trials are: a) 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 (number of inoculations, dilutions of the tuberculins, statistical program, infection or sensitization, period of time); and b) molecular characterization of the *M. tuberculosis* complex strains is an useful tool for epidemiological studies therefore the assignation of profiles should be standardize all over Europe.

Objectives: To organize two ring trials for all the NRLs: a) Evaluation of the methodology for determination of biological potency of the PPD in guinea pigs. The participants for this ring trial will be the NRLs which perform this protocol together with the European manufacturers. PPDs will be sent (European/International Standard and blinded PPDs) to determine the biological potency in each laboratory/company; b) Evaluation of the molecular characterization of *M. tuberculosis* complex strains. A set of DNA will be sent to NRLs to assign a SB number by DVR-spoligotyping technique (with commercial or EU-RL membranes).

Expected outputs: a) Knowledge of the methodology for determination of the biological potency of tuberculins in guinea pigs and comparison of results; b) Proper assignation of the spoligotyping profiles in order to compare the results around Europe.

5. Applied technologies (MALDI TOF MS) for the diagnosis of bovine Tuberculosis.

Description: The VISAVET Health Surveillance Centre has recently acquired a Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI TOF MS) system that will be incorporated both in the routine diagnostic activities as well as in highly innovative research activities since this technology is low cost, easy operation, large-scale sample analysis and promptitude of results.

Objectives: a) Set up a protocol for the identification of mycobacterial species of veterinary interest. A systematic comparative study will take place throughout 2014; b)

Analysis of PPDs. The European standard stored under different conditions will be analyzed by MALDI TOF MS in different time points in order to identify variations in the content and thus the quality of the product. Moreover, MALDI TOF MS profiles of the major commercially available bovine PPDs will be compared in an attempt to identify variations in the content of each commercial product that could affect its potency; and c) Detection of IFN-γ. In an innovative approach, the EU-RL will try to detect and quantify the produced interferon gamma in the plasma of stimulated blood lymphocytes by exploiting the high analytical sensitivity and accuracy of the MALDI TOF MS in the detection of proteins.

Expected outputs: a) Identification of mycobacterial species by a MALDI TOF MS system; b) Determination of the content and quality of PPDs by MALDI TOF MS system; and c) Detection and quantification of IFN-γ in plasma by MALDI TOF MS system.

6. Reference reagents.

6.1. European Standard.

Description: The international standard (IS) (Health Protection Agency, United Kingdom) is used as a control in the *in vivo* testing of the tuberculins although its stock is limited. For this reason, one of the objectives for the EU-RL is the production of an European Standard for *in vivo* testing of the tuberculins. So far, preservation studies to define the best conservation systems and studies in guinea pigs have been carried out by the EU-RL for Bovine Tuberculosis.

Objectives: The main objective would be the production of an European Standard tuberculin to be distributed to the stakeholders for their potency testing studies to avoid consuming the IS stock. During 2014, 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. This European Standard will be also included in the ring trial.

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

6.2. Other reference reagents.

Description: As defined in the Annex II of the Commission Regulation (EC) No 415/2013 one of the tasks of the EU-RL is to prepare, control and supply reference reagents to the National Reference Laboratories in order to standardise the tests and reagents used in the Member States.

Objectives: a) To set up a Mycobacteria strain panel (DNA) for the organization of the DVR-spoligotyping ring trial; and b) To design a VNTR marker for the most problematic loci.

Expected outputs: a) Mycobacteria strain panel to organize the molecular characterization ring trial; and b) VNTR marker to assign properly the MIRU-VNTR profiles.

7. World Wide Web page

Description: The EU-RL website (www.bovinetuberculosis.eu) is the gateway to all NRLs, EC and Scientific Community to all the activities performed by the EU-RL for Bovine Tuberculosis. It includes the list of NRLs, activities, databases, documents, EU-RL contact details and links. The objective over the years is to improve the website including new sections if necessary (ie. in 2013 an on-line system for the registration and participation in the comparative test was designed).

Objectives: a) Maintenance of the website; b) To develop an online system to include the new papers related to diagnostic techniques for bovine tuberculosis (information will be obtained from PubMed.gov). An automatically e-mail will be sent to all NRLs/EC when a new paper is published and will also be included in the "EURL Databases" section ("BT Publications"); and c) To complete the "Bovine TB" section regarding the disease (aetiology, pathology, diagnosis, molecular characterization, etc.).

Expected outputs: a) On-line system for a periodic update of new scientific papers regarding diagnosis of bovine tuberculosis; and b) "Bovine TB" section.

8. Missions

Description: If requested by the EC or under specific circumstances, the EU-RL staff will visit the European Commission as well as the NRLs dependencies. Moreover, the EU-RL staff visits: a) cattle farms to perform field studies (IFN-γ, serology, potency studies) and collect samples (blood); 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 in surveillance, epidemiology and prevention 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 (ie. 6th International Conference on *M. bovis*, United Kingdom; 35th Annual Congress of ESM, Austria) and training courses.

Expected outputs: a) Technical advice to 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

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 learn 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. Meeting.

Description: Standardisation of molecular characterization protocols as well as nomenclature for assigning the profiles to the *M. tuberculosis* complex strains is critical to be able to perform epidemiological studies all over Europe. Nowadays there are two main databases: The SITVIT Database (Public Health, Demay et al. 2012) and mbovis.org (Animal Health, Smith et al. 2012). Since tuberculosis is a zoonosis it should be a common database with *M. tuberculosis* complex isolates from human and animal (cattle, goats, sheep, wildlife, zoo animals, pets, …) origins to be able to compare and study the source of the outbreaks, dissemination of profiles, etc. For this reason, the EU-RL will work towards the unification of both databases and would propose a common meeting with the responsible of both databases to study the possibility of joining/linking both databases.

Objectives: To organize a meeting with the responsible of the two databases of *M. tuberculosis* complex strains.

Expected outputs: Set up the type of collaboration between the existing databases compiling molecular characterization of *M. tuberculosis* strains in Europe.

B. OTHER ACTIVITIES OF THE EU-RL FOR BOVINE TUBERCULOSIS FOR 2014.

The following tasks will remain permanent activities of the EU-RL for 2014.

1. Preparation, control and supply of reference reagents, and protocols.

2. Collection of representative samples of *Mycobacterium* spp.

3. Collection of representative serum/plasma recovered from infected animals.

4. Isolation, identification and typing of *Mycobacterium* spp.

5. Supply of the home-made spoligotyping membranes.

6. Technical assistance to the Commission and NRLs and participation in the bovine tuberculosis subgroup of the Task Force.

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

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