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**Working Document**  
**concerning the WORK PROGRAMMES**  
**of the COMMUNITY REFERENCE LABORATORIES**  
**in the field of animal health and live animals**  
**for 2009**

### **3.15 WORK PROGRAMME FOR THE COMMUNITY REFERENCE LABORATORY FOR BOVINE TUBERCULOSIS, 2009**

#### **I. LEGAL FUNCTIONS AND DUTIES**

The legal functions and duties of the CRL for bovine tuberculosis are established in Annex II to Commission Regulation (EC) No 737/2008 of 28 July 2008 designating the Community reference laboratories for crustacean diseases, rabies and bovine tuberculosis, laying down additional responsibilities and tasks for the Community reference laboratories for rabies and bovine tuberculosis and amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council (OJ L 201, 30.7.2008, p. 29–32).

#### **II. OBJECTIVES FOR THE PERIOD JANUARY – DECEMBER 2009**

1. Coordination of the methods used in the Member States for the diagnosis of bovine tuberculosis. These tasks are intended to cover (1) and (2) of Annex II Commission Regulation (EC) No 737/2008 of 28 July 2008 designating the Community reference laboratories for crustacean diseases, rabies and bovine tuberculosis, laying down additional responsibilities and tasks for the Community reference laboratories for rabies and bovine tuberculosis and amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council (OJ L 201, 30.7.2008, p. 29–32). .
  - a. Isolation and identification of *Mycobacterium* spp. Duration: yearly, expected to be continued during following years.
    - Bacteriology diagnosis of *Mycobacterium* spp. from clinical samples from domestic and wild animals will be available for Member States. Bacterial culture remains the “gold standard” method for confirmation of infection. In addition, microscopic examination is useful for a presumptive confirmation of acid fast bacilli.
    - Culture procedure will be carried out in bio-safety level 3 laboratories using samples from domestic animals and other species, including wildlife. Both solid media and liquid media will be used. Identification of isolates will be carried out based on DNA extraction and subsequent amplification by Polymerase Chain Reaction (PCR) targeting genus, species or complex-specific sequences. Sequencing may be used to characterise key genes.
  - b. Typing *Mycobacterium* spp. strains. Duration: yearly, expected to be continued during following years.
    - Molecular epidemiology is an integration of conventional epidemiology with molecular techniques to track specific strains of pathogens in order to understand the distribution of disease in populations. Molecular typing of

isolates has become a valuable tool in the study of tuberculosis epidemiology allowing investigators to detect outbreaks and achieve better knowledge of transmission and increased incidence of infection.

- A wide variety of DNA-fingerprinting techniques have been developed to differentiate the *M. tuberculosis* complex isolates and epidemiological purposes. PCR-based fingerprinting techniques have been implemented in most laboratories such as the direct variable repeat (DVR)-spoligotyping. Nowadays there is a website that hosts the spoligotype database of *Mycobacterium bovis* and *Mycobacterium caprae* strains ([www.mbovis.org](http://www.mbovis.org)).
  - The mycobacterial interspersed repetitive units (MIRU)-variable number tandem repeat (VNTR) typing has also been developed to increase the discrimination of the *M. tuberculosis* complex species. The MIRU-VNTR technique is based upon repeat number polymorphism within some tandemly repetitive DNA sequences. However, few epidemiological studies have been performed. Some of the most polymorphic loci studied are VNTR 3232, ETR-A, ETR-B, MIRU-26, QUB11a, QUB11b, ETR-C, MIRU-4, among others, and a combination of them generates a MIRU-VNTR genotype which define the isolate. Nevertheless, the degree of discrimination of each locus not only depends on the locus but also on the geographical origin of the samples.
  - *Mycobacterium* spp. submitted by National Reference Laboratories will be characterized by molecular tools that would be updated according to scientific literature and international acceptance. Initial choice would be DVR-spoligotyping and additional MIRU/VNTR analysis.
  - Regarding equipment, VISAVET has all the equipment necessary to perform the molecular techniques (DVR-spoligotyping, Restriction Fragment Length Polymorphism, Variable Number Tandem Repeat and Pulsed-Field Gel Electrophoresis). The sequencing service required for Multilocus sequence typing, description of polymorphisms, etc. will be outsourced to a company specialized in ultra high throughput DNA sequencing. Interpretation of the characterization results will be performed with the Bionumerics software (Applied Maths) which consists of the Basic Software and 5 modules (Cluster analysis and Phylogeny, Identification and Library Manager, Comparative Quantification and Polymorphism Analysis, Dimensioning techniques, and Database Sharing Tools).
- c. A *Mycobacterium* spp. culture collection. Duration: yearly, expected to be continued during following years
- A collection of *Mycobacterium* spp. causing tuberculosis in animals will be organized and maintained. This collection will be mainly composed of *M. bovis* and *M. caprae* isolates from domestic and wild animals, but include as well other important veterinary pathogens such as *Mycobacterium avium* subsp. *paratuberculosis*, *M. a.* subsp. *hominissuis* and atypical mycobacteria. These isolates will be used for evaluation of bacteriology-based diagnosis and

molecular characterization. Isolates will be supplied to National Reference Laboratory upon request.

- d. Database of strains isolated across the Community. Duration: yearly, expected to be continued during following years
- A database of *M. bovis* and *M. caprae* isolates obtained in all countries belonging to the EU will be set up. This database will contain enough information to provide a valuable epidemiological use, i.e. potential traceability of isolates. For this purpose, isolates will be characterised by DVR-spoligotyping and MIRU/VNTR typing according to standardised methodology. Isolates will be assigned a code, and authoritative profile, and stored with information regarding animal species and geographical origin. This information can be provided by the National Reference Laboratories and/or by the CRL. The access to the database will be restricted; guidelines and use to be defined at a workshop.
- e. Validation of reagents to be used in immunological tests. Duration: 1) validation of reagents: yearly, to be continued during following years; 2) specific research: a limited period ( expected 102 months).
- A key task of the CRL will be the validation of tuberculin (Purified Protein Derivative, PPD) and antigens submitted by National Reference Laboratories. These reagents are basic to immunology-based tests that are used for in vivo diagnosis. Skin tests are the main techniques used worldwide as the official diagnostic tests in the eradication programmes. Large differences among potencies have been described depending on manufacturer and batches.
  - The potency of tuberculins will be assayed in the guinea pig model and in cattle. Also alternative system to reduce animal experiments and improve animal welfare will be evaluated, i.e. the evaluation in antigen-sensitised rather than infected animals. Tuberculin test in guinea pigs: the potency of tuberculin is determined by comparison with a reference preparation of bovine/avian tuberculin (known potency) in guinea pigs sensitised with *M. bovis* (bovine PPD) or *M. avium* (avian PPD). To meet the requirements of statistical analysis each tuberculin is used at three dilutions (1:200; 1:1000 and 1:5000, for bovine PPD).
  - Potency assay of batches of routine use bovine and avian PPD will be carried out at the level 3 bio-safety laboratory as is indicated in Chapter 2.3.3 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE). In each assay, the potency will be estimated by assay against an International Standard bovine/avian tuberculin. The International standard bovine tuberculin is a freeze dried preparation that is produced and calibrated at Central Veterinary Institute, Lelystad. It has a potency of 32.500 international units per mg, and is used also at three dilutions. The three bovine PPDs under assay are also used at those three dilutions. For avian PPD the standard is diluted at 1:80, 1:400 and 1:2000 and the PPD to test at 1:100, 1:500 and 1:1250. The diluted

PPDs will be inoculated into each animal in four sites on each side of the back. The different diameters of erythema will be measured with callipers in millimetres and recorded on assay sheets. The results will be evaluated using standard statistical methods. The relative potencies of the test tuberculin will be calculated with 95% confidence limits. According to the European Pharmacopoeia, the estimated potency for bovine tuberculin must be not less than 66% and not more than 150% of the potency stated on the label (between 75% and 133% for avian tuberculin).

- Tuberculin test in cattle: the potency of a tuberculin is estimated by comparing the size of the reaction elicited by an intradermal inoculation, and comparison to the size of the reactions of a “standard” tuberculin of known potency. To meet the requirements of statistical analysis each tuberculin is used at two dilutions, usually, at normal strength (which for many tuberculin is 1mg/ml) and 20% of normal strength.
- To determine the potency of batches of bovine PPD, the VISAVET Laboratory will carry out the assay in a level 2 bio-safety farm housing cattle giving positive result to a single intradermal comparative tuberculin test (SICTT). In each assay, the potency of three routine use batches is estimated by assay against an International Standard bovine tuberculin (CVI, Lelystad) used at dilutions of 1 mg/ml and 0.2 mg/ml. The three bovine PPDs under assay are also used at dilutions of 1 mg/ml and 0.2 mg/ml. The tuberculin preparations will be inoculated into each animal at four sites on each side of the neck according to assay worksheets. The skinfold thickness at the injection sites will be measured and recorded on the worksheets. A statistical analysis of the increase in skinfold thickness at each site will be done to estimate the potencies.
- The PPDs and antigens will be also evaluated in the interferon-gamma assay (IFN- $\gamma$ ). This in vitro test, also measuring the cellular mediated immunity, is based on the detection of IFN- $\gamma$  in plasma supernatants from tuberculin-stimulated whole blood culture. Comparative evaluation of different PPDs and antigens will be carried out in blood from infected cattle and goats housed at the research farm and/or field trials. The true infection status to determine sensitivity and specificity will be determined by post-mortem studies (presence of macroscopic lesions and samples will be collected for culture of mycobacteria).
- Additionally, a number of field trials will be developed to focus on specific concerns related to immunological diagnostics. First one is the potential interference of *Mycobacterium avium* paratuberculosis (Map) infection in the sensitivity of the IDTB and IFN- $\gamma$  tests in cattle and goats naturally co-infected with *M. bovis* or *M. caprae*. This interference has been recently pointed out by some reports but further studies are necessary to verify this effect under a variety of field conditions and to understand its impact on the results of the eradication campaigns. Second concern is the performance of the diagnostic tests in young animals, specifically calves under 42 days of age that are not

subjected to pre-movement testing but may be a source of infection via trade. Young animals may show erratic responses to antigens and even calves born to infected cows may be anergic. Third concern relates to the effect of the delivery of tuberculin depending on the use or misuse of some injection systems.

f. Harmonisation of protocols.

- If deemed necessary by the Commission and National Reference Laboratories, the CRL for bovine tuberculosis can collect and collate information from National Reference Laboratories to design, edit and distribute procedure manual on standard test methodologies in order to harmonise protocols. This series could include i.e. “Procedure manual for sampling and submitting clinical samples for microbiological culture”, “Procedure for microbiology identification of bovine tuberculosis”, “Procedure for molecular characterization for epidemiological purposes at EU level”, “Procedure manual for the performance of the single intradermal comparative cervical tuberculin test and the gamma-interferon”, “Epidemiological survey for the investigation of outbreaks of bovine tuberculosis”, etc...

g. Preparation and control of reference reagents. Duration: yearly, expected to be continued during following years.

- The CRL will prepare and control the reference reagents in order to standardise the protocols used in the different countries and to validate the ring trials. Also, reagents submitted by National Reference Laboratories will be evaluated. Substances, reagents and other biological materials available or prepared at VISAVET will also be available to National Reference Laboratories for harmonisation of protocols; i.e. *Mycobacterium bovis* BCG (Danish) CCUG 27863 (internal reference code VV-E-457), *Mycobacterium avium* subsp. *avium* CCUG 20992 (ATCC 25291) (VV-E-480), *Mycobacterium tuberculosis* CCUG 37357 (H37Rv) (VV-E-481), *Mycobacterium avium* subsp. *paratuberculosis* CIP 103963 (VV-E-523), recombinant Bovine IFN-gamma RYD-2300-BG-025 (08/0737) or bovine plasma in lithium heparin IBN-N-12 (08/0868).

h. Comparative tests. Duration: yearly, expected to be continued during following years.

- A main activity of the CRL for bovine tuberculosis will be the organisation of periodic ring trials for standardization of techniques and setting up or harmonisation of different protocols at the National Reference Laboratories. This will include the design of the ring trial, the preparation, labeling and shipping of material, collection of the results from the participants, analysis of results and reporting.
- Need and priorities for these ring trials will be discussed in the first workshop (2008).

i. International standards and practices. Duration: yearly, expected to be continued during following years.

- All samples received will follow a reliable system of procedures for dispatching and receiving samples (including infectious material) between laboratories that has been established at VISAVET. The system is useful to track the samples since they enter the laboratory until their elimination or preservation. The laboratory only carries out the assays requested and does not take part in the sampling. All this information is enclosed in a general procedure (Samples management. Entries, identification and traceability PG/008/VV. Gestión de muestras. Entradas, identificación y trazabilidad).
- The traceability is based on a correct identification of a sample through every stage of the process. Therefore a minimum data for the sample identification is necessary (entry code, reference, customer, department or laboratory, specific method, dates, result and report). This will take into account the large variety of material expected to be received which includes clinical samples for culture, identification and molecular characterisation; solid or liquid media with positive growth for DNA extraction, identification and molecular characterisation; DNA extracted for identification and molecular characterization; patterns from molecular characterisation, PPDs for potency evaluation; sera or plasma for interferon-gamma ELISA tests, etc...
- The outstanding protocols that will be applied at the CRL for bovine tuberculosis will be implemented according methodology to UNE-EN ISO/IEC 17025 ("General requirements for the competence of testing and calibration laboratories") following the current laboratory methods (PE: specific method) used in the VISAVET Mycobacteria Group for the detection, identification and characterization of mycobacteria.

j. Keeping abreast of developments. Duration: yearly, expected to be continued during following years.

- Staff of the CRL for bovine tuberculosis will keep abreast of developments in surveillance, epidemiology and prevention of tuberculosis throughout the world. This will include as well the potential use of vaccines under certain circumstances and new uses of products of veterinary immunology. To fulfil this commitment, the members of the Laboratory will get information through different ways (scientific papers in national or international journals, attendance to congresses, and workshops, specific training courses, reports from experts, legislation, etc.) and also through active participation in research projects. The relevant information will be distributed to the National Reference Laboratories.

k. Dissemination. Duration: yearly, expected to be continued during following years.

- The existence, role and tasks of the CRL for bovine tuberculosis will be disseminated via national and international routes to several levels. The information will be disseminated mainly through:

- Presentations at international and national congresses or conferences, publication in international and national journals;
  - World Wide Web page which will contain basic updated information.
2. Organisation of workshops and training of personnel. This task is intended to cover (3) of Annex II SANCO/01240R1/2008. Commission Regulation (EC) No 737/2008 of 28 July 2008 designating the Community reference laboratories for crustacean diseases, rabies and bovine tuberculosis, laying down additional responsibilities and tasks for the Community reference laboratories for rabies and bovine tuberculosis and amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council (OJ L 201, 30.7.2008, p. 29–32).
  3. A workshop to will be organized in 2009. Participants (32) will include a member from each National Reference Laboratories in the Member States as described in the Commission Regulations. Subjected to Commission approval, a member from Institutions participating in a network on Mycobacteria of veterinary interest “VENoMYC” (funded by the EU) would be invited. VENoMYC is a multidisciplinary network made up with 37 partners from 17 countries that were selected on basis of active researching on mycobacterial infections. Duration: yearly, expected to be continued during following years. For the workshop, an agenda including some of the following sections and/or additional material or information, will be distributed to all partners by electronic mail, or other media if required:
    - Short lectures of the topic;
    - Hands-on training in the laboratory;
    - Discussion with the information presented in the workshop;
    - Visit to farms and/or other institutes of importance for the issue in study.
    - A report according to rules defined by the Commission would be prepared.
    - Training of experts from the Member States and from third countries (when appropriate) will be performed by organisation of short courses or by individual training. These short visits (1-2 weeks) will be open to all National Reference Laboratories to allow the establishment of new protocols and techniques in their laboratory of origin. Afterwards, the trainee will be requested to submit a brief report.
    - All the personnel under training will carry out a training period before working in the level 3 laboratories. The unit responsible for the security and safety in the laboratory will ensure that all the protocols are performed correctly. In the VISAVET Quality Manual (Manual de Calidad del Laboratorio de Vigilancia Sanitaria) is detailed that all the new personnel should follow a training period established for each protocol in the training programme.
  4. Technical assistance to the Commission. This task is intended to cover (4) of Annex II Commission Regulation (EC) No 737/2008 of 28 July 2008 designating the Community reference laboratories for crustacean diseases, rabies and bovine

tuberculosis, laying down additional responsibilities and tasks for the Community reference laboratories for rabies and bovine tuberculosis and amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council (OJ L 201, 30.7.2008, p. 29–32). .Duration: yearly, expected to be continued during following years.

- The staff of the CRL for bovine tuberculosis will be accessible to provide technical assistance to the Commission and upon its request this will be extended also to its Institutions. The staff could also provide support to Member States on specific issues regarding eradication programmes. A contact with Public Health Institutions would be established in order to increase awareness of the zoonoses.
  - Staff will participate in international fora relating bovine tuberculosis i.e. the coming Fifth International Colloquium in Bovine Tuberculosis (August 2009).
5. Research activities. This task is intended to cover (5) of Annex II .Commission Regulation (EC) No 737/2008 of 28 July 2008 designating the Community reference laboratories for crustacean diseases, rabies and bovine tuberculosis, laying down additional responsibilities and tasks for the Community reference laboratories for rabies and bovine tuberculosis and amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council (OJ L 201, 30.7.2008, p. 29–32). Duration: expected to be maintained at least during following four years.
- The CRL for bovine tuberculosis will maintain its active research directed towards the improved control and eradication of bovine tuberculosis through:
    - Collaboration with National Reference Laboratories, i.e. relevant problems associated to local farming practices on the epidemiology of the infection in the Member States (livestock breeding systems and specific role of wildlife) and impact on detection of infection in animals; and carrying out validation trials;
    - Analysis of the information collected and preparation of reports associated to the activities of the CRL.
  - The CRL for bovine tuberculosis will maintain research activities at international level participating in the following projects:
    - Veterinary Network of Laboratories Researching into Improved Diagnosis and Epidemiology of Mycobacterial Diseases, VENoMYC. Co-ordination Action SSPE-CT-2004-501903. 2004-2008. Co-ordinator: Lucas Domínguez.
    - This Co-ordination Action is addressing the most relevant problems (lack of appropriate methods of diagnosis; the role played in the epidemiology of the diseases by other domestic and wild animals; difficulties in the laboratory work with these pathogens; and lack of adequate vaccines that do not

interfere with diagnosis) regarding diagnosis of mycobacterial diseases. In addition, application of new systems is included, i.e. use of functional genomics to detect new molecular markers and/or to develop new vaccines. The main approach of this Co-ordination Action is to share technology and expertise in order to both avoid research fragmentation and obtain a common knowledge on mycobacterial diseases. The final target is to develop harmonized recommendations and/or procedures for their potential transposition into EU policies.

- Development of improved tools for detection of paratuberculosis in livestock, *M. paratuberculosis* in food and for the assessment of the risk for human exposure, ParaTBTools. FP6-2004-FOOD-3B. 2006-2009. Co-ordinator: Douwe Bakker. The overall strategic objective of this research project is to generate new tools for the diagnosis and detection of *M. a. paratuberculosis* in animals and animal products; to improve methods for elimination of *M. a. paratuberculosis* from foodstuffs; and to define the risks associated with *M. a. paratuberculosis* and its potential role in Crohn's disease. The VISAVET Mycobacteria Group is involved in 6 workpackages (WP1: Standardisation and harmonisation of reagents and protocols; WP2: Identification and characterization of novel antigens; WP3: Development of improved methods; WP4: The Use of a cattle infection model for *M. a. paratuberculosis*; WP5: Associations between ruminant immune responses and pathology; and WP6: Information dissemination).
6. Strategies for the eradication of bovine tuberculosis, TB-STEP, FP7-KBBE-2007-1. On negotiations. Co-ordinator: Lucas Dominguez. This project plans a multifaceted battlefront to approach the eradication of bovine tuberculosis. The consortium is made up of 12 partners from eight countries which will research on eight workpackages devoted to improved tools and to develop strategies for the eradication of bovine tuberculosis in areas where the disease is present in both domestic and wildlife populations. It will include: 1) vaccination of bovine animals and wildlife, (2) control of populations to reach numbers compatible with animal welfare and strategies to limit the contact between domestic and wild species, and (3) the development of improved diagnostic tools for detection of infected animals.