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Working Document

on the WORK PROGRAMMES

of the COMMUNITY REFERENCE LABORATORIES

in the field of animal health and live animals

for 2010

3.15 WORK PROGRAMME FOR THE COMMUNITY REFERENCE LABORATORY FOR BOVINE TUBERCULOSIS, 2010

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. Isolation and identification of Mycobacterium spp.

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.

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

2. Typing Mycobacterium spp. strains.

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. 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 characterised 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. Spoligotyping numbering will follow the code number from the website that hosts the spoligotype database of *Mycobacterium bovis* and *Mycobacterium caprae* strains (www.mbovis.org). MIRU/VNTR analysis will follow the protocol agreed at the VENoMYC network.

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 characterisation 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).

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

3. A Mycobacterium spp. culture collection.

A collection of *Mycobacterium* spp. causing tuberculosis in animals is being organized and maintained. This collection is 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 characterisation. Isolates will be supplied to National Reference Laboratory upon request.

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

4. Database of strains isolated across the Community.

A database of *M. bovis* and *M. caprae* isolates obtained in all countries belonging to the EU would provide interesting information to be used in tracing international transmission. For this purpose, isolates should be characterised by DVR-spoligotyping and MIRU/VNTR typing according to standardised methodology and stored with information regarding animal species and geographical origin. However, this proposal needs further discussion about guidelines and participation by the National Reference Laboratories.

Duration: to be discussed again at the workshop, expected to be continued during following years.

5. Validation of reagents to be used in immunological tests.

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.

According to information kindly provided by NRLs, there are several tuberculin preparations in use in Europe. Because of the number of different products (different manufacturers), a selection will be tested. 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 then 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 indicated in Chapter 2.4.7 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2009 (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-γ). This *in vitro* test, also measuring the cellular mediated immunity, is based on the detection of IFN-γ 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 continued 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-γ 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.
Duration: 1) validation of reagents: yearly, to be continued during following years; 2) specific research: a limited period (expected 24 months).

6. Harmonisation of protocols.

The CRL for bovine tuberculosis will 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 characterisation 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…

7. Preparation and control of reference reagents.

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

8. Comparative tests.

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 were initially discussed in the first workshop (2008).

Two main options are available: 1) an evaluation of performance of the IFN-γ ELISAs in the laboratories, and 2) direct diagnosis by PCR (from clinical and /or spiked tissue samples). The comparative test to be carried out during 2010 will be decided by NRL representatives at the workshop in November 2009.

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

9. International standards and practices.

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 characterisation; 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 characterisation of mycobacteria.

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

10. Keeping abreast of developments.

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.

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

11. Dissemination.

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.

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

12. Organisation of workshops and training of personnel.

This task is intended to cover (3) of Annex II to Commission Regulation (EC) No 737/2008.

The CRL organized a first workshop (Madrid, December 2008) as an introductory meeting in which topics included in the programme were covered briefly. A second workshop has been scheduled for November 2009. Thus, no workshop has been foreseen for 2010, and this task would be continued again in 2011.

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

13. Technical assistance to the Commission.
This task is intended to cover (4) of Annex II to Commission Regulation (EC) No 737/2008.
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 Director of the CRL will participate in the bovine tuberculosis subgroup of the Task Force. 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.
A number of missions have been forecast, which include the Commission and NRLs in Spain, the UK and the Republic of Ireland.
Duration: yearly, expected to be continued during following years.

14. Research activities.
This task is intended to cover (5) of Annex to Commission Regulation (EC) No 737/2008.
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 has addressed 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. The main approach of this Co-ordination Action was to share technology and expertise in order to both avoid research fragmentation and obtain a common knowledge on mycobacterial diseases.

Though this project has completed its formal duration, partners have agreed to maintain the partnership and it may apply to future calls.

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 characterisation 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 WP16: Information dissemination).

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.

Duration: expected to be maintained at least during following four years.