French Agency for Food, Environmental & Occupational Health Safety

Maisons-Alfort

LABORATOIRE DE SANTE ANIMALE

ANIMAL HEALTH LABORATORY

Unité Zoonoses Bactériennes

Bacterial Zoonoses Unit

Centre National de Référence des *Brucella*

National Reference Centre for Human Brucellosis

Laboratoire National de Référence pour la Brucellose, la Chlamydiose, la Fièvre Charbonneuse, la Morve, la tuberculose & la Tularémie animales

Animal Anthrax, Brucellosis, Chlamydiosis, Glanders, Tuberculosis & Tularaemia National Reference Laboratory

Laboratoire de Référence O.I.E. / FAO pour la Brucellose, et la Tuberculose bovine

OIE /FAO Reference Laboratory for Brucellosis, and Bovine Tuberculosis

Laboratoire de Référence de l'UE pour la Brucellose

EU Reference Laboratory for Brucellosis

Laboratoire de Référence de l'UE pour les Maladies Equines (Morve)

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2015 Work Programme of the EU Reference Laboratory for Brucellosis

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Introduction

The **Laboratoire de Santé Animale de Maisons-Alfort** (*Animal Health Laboratory*) of **ANSES** (*French Agency for Food, Environmental and Occupational Health Safety*), formerly the LERPAZ laboratory (*Animal Diseases & Zoonoses Research Laboratory)* of AFSSA (*French Food Safety Agency*) was designated by the Commission Regulation (EC) No 776/2006 of 23 May 2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards Community Reference Laboratories, as the Community Reference Laboratory (CRL) for Brucellosis *(and late 2009, the name CRL became EURL)*.

Work programme 2015

The following activities are foreseen for 2015:

Activity 1. *Support to DG Sanco and to EU Brucellosis NRLs*

Activity 2. *Inter-laboratory ring-trials*

Activity 3. *NRLs network related training activities and animation*

Activity 4. *Development, evaluation of innovative analytical methods*

The details of the estimated budget per activity are provided in the attached table. The objectives and expected outputs per activity are as follows:

Activity 1. *Support to DG Sanco and to EU Brucellosis NRLs*

This activity includes in particular the following permanent activities:

1.1. Studies on sera presenting unexpected or doubtful results;

1.2. Identification and biotyping of *Brucella* strains (when the NRL is unable to fully identify/biotype the strain or in case of atypical strains);

1.3. Supplying available field or reference *Brucella* strains and standardised reagents for *Brucella* typing;

1.4. Supplying available standardised reagents for brucellosis immunological diagnosis;

1.5. Control of diagnostic antigens or kits (EU official tests only) according to EU or OIE standards;

1.6. Control of national secondary standards as adequately standardised against international primary standards;

Sub-activities (1.1) *to* (1.6) *cannot be planned in advance since it depends on the NRLs and DG-Sanco requests.*

1.7. Establishment of Standardised Technical procedures at the EU level;

In 2015, a standard operating procedure (SOP) concerning *Brucella* identification (species/ biovar) will be developed.

1.8. Collection of representative samples of *Brucella* strains isolated in the EU and maintenance of the collection;

To complete the strain collection dedicated in 2014 to *B. melitensis* biovar 1 and 2 (biovar 3 collected in 2013), it is foreseen in 2015 to organise the collection of *B. abortus* strains in the concerned NRLs, in relationship with

their country epidemiological context. These strains will be biotyped in details before inclusion in the EURL *Brucella* collection.

1.9. Report of the Brucellosis EU NRLs' annual activity.

A questionnaire will be achieved after a consultative presentation during the next workshop (September 2014) and launch at the beginning of 2015. The information data will be summarised and communicated to the whole network during the 2015 Workshop as well as DG SANCO.

The objective of the survey has been slightly revised and the focus will be put on :

- NRLs activity assessment (toward their own national network) in order to identify ways of improvement and to share examples of good practices,
- EURLs networking activities according to NRLs expectations, in order to improve EURL support and address issues of common concern.

To summarize, the main deliverables from activity 1 will be evaluated through the following outputs and criteria:

✓ **Expected outputs :**

- Positive and doubtful cases will be confirmed or unvalidated
- Biotyping of strains will be performed upon request
- *Brucella* reference and field strains and standardised reagents for *Brucella* typing and / or brucellosis immunological diagnosis will be provided to NRLs upon request, according to EURL supplying constraints
- Diagnostic antigens, kits (EU official tests only) or secondary standard sera will be controlled according to EU or OIE standards upon request
- A standard operating procedure (SOP) concerning *Brucella* phenotypical identification (species/ biovar) will be conceived and communicated to NRLs
- Strain collection will be completed according to NRLs' participation
- A survey will be organized to record NRLs activities (toward their own national network) and expectations

✓ **Performance indicators :**

- Number of confirmed and unvalidated cases
- Number of strain identifications
- Number of biological reagents supplied to NRLs
- Number of biological controls performed
- Number of NRLs participating to the survey.

Activity 2. *Inter-laboratory ring-trials*

One inter-laboratory ring-trial (ILPT) is foreseen for the 1st semester of 2015. This ILPT will focus on the performance of milk ELISA in bovine brucellosis (Milk- iELISA). The objective of ILPT is to evaluate the progress made by EU NRLs in performing this test prescribed in dairy cattle for the control/surveillance/eradication programmes in the EU. A similar proficiency ring-trial was organised in 2013, in 2011-2012 and in 2008-2009. This ILPT will give the EURL the opportunity to evaluate the improvement of the indirect diagnostic performance through the EU NRL network, in particular for NRLs who showed some difficulties during the last ILPTs.

To summarize, the main deliverables from activity 2 will be evaluated through the following outputs and criteria:

- ✓ **Expected outputs:**
  - One ILPT organized for NRLs regarding the performance of milk ELISA in bovine brucellosis,
  - Good quality of proficiency test restitution.
- ✓ **Performance indicators:**
  - Number of NRLs with satisfactory ILPT results,
  - Reduction in the number of NRLs failing compared to previous trials
  - Number of positive satisfaction surveys for the ILPT.

Activity 3. *NRLs network related training activities and animation*

3.1 Training activities :

The EURL plans to organise two training sessions in 2015, *i.e.*:

- A 2-day training session on "Identification and typing of *Brucella* spp.". The training programme will be adjusted depending on NRLs needs which will be interactively discussed during the next 2014 workshop. For bio-safety and bio-security reasons (work in BSL3 facilities), this session will be limited to 8 NRLs (one participant per NRL) with a priority given to NRLs having faced difficulties during 2012-2013 inter-laboratory ring-trial;
- A 2-day training session on "Brucellosis serological diagnosis". The training programme will be adjusted depending on NRLs needs, which will be discussed during the 2014 workshop. The training will be dedicated either to EU standardised Complement Fixation test or to biologicals control (especially RB antigen control). This session will be open to 8 NRLs (one participant per NRL).

The objective is to evaluate the technical skills of the EU NRLs regarding direct and indirect diagnosis of *Brucella,* and to help some NRLs in implementing the EU SOPs especially in indirect diagnosis of brucellosis.

While priority will be given to EU member states NRLs, these training sessions will be open to EFTA, EU-candidate and Balkans countries having faced difficulties in previous corresponding ring-trials. For non-EU countries, training/accommodation fees will be taken care of by the corresponding NRL. When appropriate, the eligibility for reimbursement will be evaluated by the Commission.

3.2 Workshop :

A one and a half-day workshop will be organised on the premises of the EURL during fall 2015, in order to present, share and discuss with all EU NRLs following topics:

- the EURL activity report and the future 2016 work programme being proposed to NRLs,
- the results and the analysis of the one ring-trial organised by the EURL since the workshop held in Berlin in September 2014, *i.e.* the ring-trial on milk tests planned to be launched early in 2015,
- an update on innovative molecular approaches (identification and genotyping).

One representative per NRL of the candidate countries (FYROM , Iceland, Serbia, Montenegro and Turkey), as well as one contact per West Balkan country (Albania, Kosovo, Bosnia-Herzegovina),will be invited to attend this workshop. A technical report of this workshop will be prepared by the EURL and sent to NRLs together with a copy of all presentations made during the workshop. As previously, the report of this workshop will be sent to DG-SANCO.

3.3 Website management :

The website development aims at facilitating NRLs access to information and exchanges between NRLs and the EURL. The website frame was achieved in 2014 and different sections regarding EURL activities and brucellosis diagnosis will be developed in 2015, with a special focus on scientific activities. All NRLs will have access to a EURL private section and general information on brucellosis and the missions of the EURL will be of public access.

To summarize, the main deliverables from activity 3 will be assessed through the following outputs and criteria:

- ✓ **Expected outputs :**
  - Training of scientists and technicians from NRLs to diagnostic tools: serology, bacteriology and molecular biology
  - High participation of NRLs for the workshop with good quality debates,
  - Updated information regarding EURL activities and brucellosis diagnostic on the website
- ✓ **Performance indicators**
  - Number of scientists / technicians trained, number of positive satisfaction surveys for the training
  - Training impact on ILPT follow-up process
  - Number of participating NRLs to the workshop; number of positive satisfaction surveys for the workshop.
  - Number of ressources available on the website: SOPs, reagent control certificates…

Activity 4. *Development, evaluation of innovative analytical methods*

4.1 To address the recommendations of EFSA for ovine and caprine brucellosis:

In order to address the recommendations made by EFSA "on a request from the Commission concerning Brucellosis Diagnostic Methods for Bovines, Sheep, and Goats" (http://www.efsa.europa.eu/en/scdocs/doc/432.pdf), this activity was launched in 2012 as regards the main recommendation concerning the Indirect and Competition Enzyme Linked Immunosorbent Assay (iELISA and cELISA) in sheep and goat brucellosis diagnosis, i.e.: " *It should be noted that, with the exception of RIDNH and BST, the new tests (cELISA1, cELISA3, FPA, iELISA1, iELISA3, and MRBT) have specificity lower compared to standard tests or not sufficiently documented (cELISA2 and iELISA2). When using Se and Sp as criteria for assessing the fitness for the purpose of intra-Community trade, it can be concluded that these new tests are not suitable for inclusion in Annex C unless new data demonstrate that these tests are at least as specific as the standard tests. Hence, studies may need to be conducted to evaluate whether changes in technical specifications may improve specificity of these new tests without compromising their sensitivity. For cELISA2 and iELISA2 it is recommended that the necessary specificity data be generated*."

The already on-going EURL study project aims at addressing these recommendations.

The project includes:

1. The collection of at least 4320 serum samples from OBmF flocks including, when possible, samples with FPSR;
2. The collection of at least 1040 serum samples from infected herds (in Southern Europe);
3. The comparative analysis of this collection in EU approved tests (RBT, CFT) as well as in, FPA, iELISA (standardised according recently adopted criteria against the OIE ISaBmS international standard serum) and cELISA of different formats (*i.e*. at least 3 different commercial kits available in the EU);
4. The analysis of test results of cELISA in terms of (*i*) sensitivity and specificity in comparison with other EU approved tests and (*ii*) efficiency as a confirmatory test in relation with the test format and the respective standardization with the international standard sera.

Steps 1-3 have been already completed. Step 3 was achieved in 2014 with FPA testing.

The step 4 is foreseen to be completed in 2015. The results will be presented during the 2015 workshop.

4.2 Development of iELISA bulk milk in small ruminants

In small ruminants dairy producing regions, the performance of a bulk milk iELISA test could be an interesting alternative to individual serology tests recommended by OIE as regards brucellosis surveillance. Indeed, bulk milk iELISA would allow an efficient serology screening on easily available samples at the herd level, while reducing the global cost of surveillance in small ruminants.

In the EU context with officially *Brucella melitensis* free (OBmF) MS and MS where an eradication program is implemented in small ruminants, bulk milk iELISA would be of interest as regards regular serology monitoring allowing a rapid detection of any seroconversion at the herd level.

Currently, most surveillance programs in small ruminants in EU (in free as well as in endemic countries) include one or two individual tests on serum (Rose Bengal test (RBT) and/or Complement Fixation Test (CFT)) which are the EU reference methods. Milk ring test is not used in small ruminants due to the particular composition of the milk. Thus no milk test is available in these species. In cattle, iELISA is widely used for a rapid, reliable and robust detection of anti-*Brucella* antibodies from bulk milk. Several commercial kits have been validated, standardized and developed in cattle and can be used for individual as well as bulk milk testing.

In this context, we propose to develop a iELISA test in sheep and goats and to evaluate its relevance for brucellosis detection in small ruminants. The study will involve a transposition of bovine milk iELISA technique in small ruminants in the laboratory. For this purpose, our project includes:

1- The collection and the test of individual and bulk milk samples from officially *Brucella melitensis* free (OBmF) flocks to adjust the specificity;

2. The preparation of artificially inoculated positive milk samples, containing various *Brucella*-antibodies concentration (mixing positive serum and negative bulk milk) to adjust the sensitivity. Considering biosafety risks associated with handling milk from infected animals with potential live *B. melitensis* strains, milk samples from infected herds will not be included in this study.

3. The adjustment and validation of a home-made iELISA prototype and protocol using existing reagents respectively commercialized in bovine milk iELISA and sheep and goat serum iELISA according to expected sensitivity and specificity.

Step 1 will be achieved by the end of 2015. Validation studies (steps 2 and 3) will be performed in 2016 and 2017.

4.3 Improvement of phenotypic and molecular tests for a more discriminative identification of *Brucella* strains, including the development of genomic approaches

Surveillance and control systems of a pathogen are based on perfect knowledge, thus an in-depth identification, of the strains circulating in the study area. To improve this knowledge on brucellosis in Europe, extensive phenotypic and molecular characterizations are highly needed.

The phenotypic and biochemical characters guide the general identification of *Brucella* genus, as well as the identification at the species- and biovar-levels. Furthermore, recent findings from the ILPT organized last year among the EU LNRs show that molecular identification is considered as an alternative or complement to established phenotypic methods, which are time-consuming, fastidious and require strict biosafety conditions.

However, the development of robust molecular tools offering a sufficiently discriminative power to differentiate strains at the species / biovar level is complicated by the very strong genetic homogeneity of the *Brucella* genus (DNA homology of >90%). To date, the most relevant genus-identification technique is a real-time PCR, which targets the genes *bcsp31*, IS*711* and *per*. Other multiplex PCR assays are available: AMOS-PCR which discriminates *B. abortus* (bv 1-2 and 4), *B. melitensis* (bv 1-3), *B. ovis* and *B. suis* (only bv 1), Bruce-ladder (8 target genes) to differentiate between the classical, vaccine and marine *Brucella* species and Suis-Ladder, modified from Bruce-Ladder to discriminate *B. suis* and *B. canis* and also the *B. suis* biovars. But with a constantly upgraded taxonomy (introduction of at least 5 new species in 7 years), these approaches may soon become obsolete.

Finer analysis, based on large-scale genome-wide data, will allow identifying some species-specific and biovar-specific molecular signatures, essential for designing new diagnostic and prognostic tools. Indeed, these novel markers will be used to help more rapid and reliable brucellosis diagnosis and they will estimate prognosis/evolution of the disease through the determination and following-up of virulence factors.

1- A representative panel of type strains and field isolates of main species circulating in EU (especially *B. melitensis* bv 1-3) collected from domestic animals, wildlife and human, will be subject to a comparative genomic analysis, based on whole genome analysis (WGS) by next generation sequencing, in order to identify potential molecular markers to be applied for *Brucella* diagnosis and to determine genes and/or mutations putatively involved in virulence.

2- In addition, strains belonging to widespread species in EU, but harbouring an atypical phenotypic pattern (*i.e.* urease negative, oxidase negative, particular agglutination...) which threaten a reliable diagnosis (*e.g*. *B. melitensis* bv 1 "Indian type" with no typical agglutination profile A+ M+++, instead of A- M+ for other *B. melitensis* bv 1 strains) will be in-depth investigated in order to determine specific molecular key features for correct identification by WGS approaches.

4.4 Molecular typing as a tool for improving the investigation of brucellosis outbreaks and human cases

Brucellosis, a worldwide zoonosis due to *Brucella* genus, has a serious public health impact and causes economic losses to animal sector. The remaining prevalence of the disease in several member states, as well as the recent outbreaks in cattle and wildlife in Belgium and France- two officially bovine brucellosis-free member states- and the high prevalence of *B. suis* bv 2 in European wildlife emphasise the need for improved surveillance tools.

Identifying genetic variants is important for the back-and-forward tracing of outbreaks (epidemiological links and sources) and is essential for surveillance programs in both human and animal health.

The current optimal tool for *Brucella* strains epidemiological genotyping is the MLVA-16 assay (Multiple-Locus-Variable-number-tandem-repeat-Analysis), which involves 16 genetic markers, organized in panels, according to their evolution speed: panel 1 (8 mini-satellites), more stable than the panel 2 micro-satellites (3 for panel 2A and 5 for panel 2B).

In other pathogens, closely related isolates could harbour some small VNTR pattern variations due to random genetic events (insertion, deletion, point mutation). These events may impact MLVA data interpretation in epidemiological investigations. In *Brucella* spp., no interpretation criteria of the similarity degree between two strains are available up to now; this complicates, and even leads to erroneous interpretation of the results within the framework of epidemiological investigations. To establish such criteria, it is essential to estimate the probability of variability at each specific locus of the considered species.

Accordingly, we propose to investigate the genetic stability of MLVA-16 markers at species and biovar levels, and its consequences for *Brucella* MLVA-data interpretation.

We propose (*i*) to compare MLVA *in vivo* patterns, *i.e.* animal and human strains, clustered in epidemiologically-related groups (same outbreak, same contamination source, laboratory-acquired infection...) and (*ii*) to develop *in*

vitro artificial systems miming the intracellular conditions (oxidative stress, acid stress, hypoxia, nutrient depletion, growth in antibiotic presence…) in order to test MLVA locus stability.

Our investigations started under the EURL 2013 work-programme and give priority to the most relevant *Brucella* species in the EU: *B. melitensis* and *B. suis* bv 2. Both *in vivo* and *in vitro* studies suggested that some pattern variations do exist between closely related *Brucella* strains which may alter data interpretation. The *in vivo* studies evidenced that 32 % of epidemiological groups showed at least one Single-Locus Variation, including 8 % with Double-Loci Variations and 12 % with multiple-loci variations (4 loci). In parallel, the *in vitro* studies suggested that the presence of antibiotics could affect the rate of genetic events, responsible for VNTR variations, in reference strains, as *B. melitensis* bv 1 strain 16M and *B. suis* bv 2 strain Thomsen.

The work will continue in 2015 to propose an in-depth definition of the MLVA locus stability, in order to determine *Brucella* specific guidelines for MLVA data interpretation.

To summarize, the main deliverables from activity 4 will be followed through the following outputs and criteria:

- ✓ **Expected outputs :**
  - Assessment of cELISA sensitivity and specificity in comparison with other EU approved tests for small ruminants (end of project : 2015)
  - Implementation of a new home-made iELISA prototype and protocol for individual as well as bulk milk testing in small ruminants (3 years project : 2015-2017)
  - Determination of new molecular signatures as novel diagnosis targets for *Brucella* strains
  - Improvement of surveillance in EU and better knowledge of *Brucella* genotypes circulating in Europe
  - Determination of *Brucella* specific guidelines for MLVA data interpretation
- ✓ **Performance indicators**
  - Scientific communication about comparative performances of small ruminant serological tests
  - Number of biological samples collected for milk ELISA validation
  - Number of *Brucella* strains investigated by MLVA-16 for marker stability study
  - *In silico* comparative analysis of genetic regions putatively involved into virulence in *Brucella*
  - Number of *Brucella* strains investigated by WGS