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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
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2016-2017
Work Programme
of the EU Reference Laboratory
for Brucellosis

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 2016 - 2017

The following activities are foreseen for 2016 – 2017:

Activity 1. *Support to DG SANTE and to EU Brucellosis National Reference Labs (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 SANTE 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-SANTE requests.*

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

One session during each annual workshop will be dedicated to review SOPs being updated on the website: in 2016, reading of SOP will be dedicated to bacteriological identification of Brucella. In 2017, reviewing of SOPs will focus on two classical serological methods (Rose Bengale Test (RBT) and Complement Fixation Test (CFT)).

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

The strain collection will be broadened in 2016 and 2017 to expand coverage of research with concerned NRLs in relation to their national 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.

As proposed in the previous EURL work programmes, a questionnaire will be annually launched, in order to assess:

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

The information data will be summarised and communicated during the next workshop to the whole network as well as DG SANTE. The 2014 questionnaire sent to NRLs will be revised according to NRL comments. Consequently a modified version will be discussed with NRLs and DG SANTE during the 2015 Workshop.

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/excluded
- Strain biotyping 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
- Strain collection will be completed according to NRLs' participation

- A survey will be annually organized to record NRLs activities (toward their own national network) and expectations

✓ **Performance indicators :**

- Number of diagnosis confirmations
- 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 proficiency proficiency-trial (ILPT) is planned to maintain a high level of performance and standardization within the NRLs network. It is proposed to focus alternatively on direct and indirect diagnostic methods : (year n) serological tests from serum samples ; (year n+1) bacteriological and molecular direct tests from suspected *Brucella* strains ; (year n+2) biological standardization controls; (year n+3) serological tests from milk samples.

The main objective of ILPTs is to evaluate the proficiency of EU NRLs for tests, respectively prescribed for the control of animal movements at EU and international levels and/or recommended for the control/eradication programmes in the EU.

For 2016, the ILPT will focus on the performance of serological tests in brucellosis (RBT, CFT, *Brucella* Standard Agglutination Test (SAT) and iELISA). Previous similar ILPTs were organized in 2012 and in 2014, with particularly satisfactory results for the latter. This fourth session will give the EURL the opportunity to evaluate the improvement stability of the indirect diagnostic performance through the EU NRL network.

For 2017, the ILPT will focus on the performance of bacteriological (identification using routine typing tests *e.g.* colonial morphology, oxidase test, urease test, CO_2 requirement...) and molecular tests (Bruce Ladder, Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA)...) on suspected *Brucella* strains. In 2012-2013, a first ring trial was organised in the whole EU as regards direct brucellosis diagnosis. Phenotypical typing was mostly partially performed whereas molecular methods, especially genus-PCR and species-PCR were well-implemented, with a high diversity of protocols. The EURL has underlined that the combination of several methods should be chosen carefully to ensure the reliability of identification and to be

consistent with routine lab work. This second session will give the EURL the opportunity to evaluate the improved identification of atypical strains using complete phenotypical typing and molecular data through the EU NRL network.

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

- ✓ **Expected outputs:**
  - One ILPT organized each year for NRLs regarding the test performance: (*i*) serological tests in 2016 and (*ii*) bacteriological and molecular tests in 2017,
  - 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 per year, *i.e.*:
In 2016, two 2-day training sessions on "Identification and typing of *Brucella* spp." will focus on both bacteriological and molecular methods with the objective of defining an adequate identification strategy. For bio-safety and bio-security reasons (work in BSL3 facilities), each session will be limited to 8 NRLs (one participant per NRL) with a priority given to NRLs having faced difficulties during 2012-2013 ILPT;
The objective is to evaluate the technical skills of the EU NRLs regarding direct diagnosis of *Brucella,* and to help some NRLs in implementing the EU SOPs.

In 2017, two 2-day training sessions will be dedicated to indirect diagnostic methods. One will be organised on "Brucellosis serological diagnosis" thus giving the opportunity to new lab workers to get trained through the EURL network. The other 2017 session will be dedicated either to EU standardised Complement Fixation test or to biologicals control (especially RBT/CFT antigen control). Each session will be open to 8 NRLs (one participant per NRL).

While priority will be given to EU member states NRLs, these training session 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.

To complete the 2-day training sessions, the EURL team (2 persons) will schedule one mission per year to provide expertise to European NRLs or to share experience on *Brucella* diagnostic methods.

3.2 Workshop :

A one and a half-day workshop will be organised during fall 2016 and 2017, in order to present, share and discuss with all EU NRLs following topics:

- EURL activity report and the future work programme being proposed to NRLs,
- results and analysis of the NRLs survey and ring-trial organised by the EURL,
- review of SOPs (see paragraph 1.7),
- update on innovative diagnostic approaches developed by EURL (see paragraphs 4.1 to 4.3)
- update from the annual *Brucella* international research conference, reporting communications and results dedicated to *Brucella* diagnostic and epidemiology,
- 2-3 presentations from different NRLs dedicated to *Brucella* diagnostic tools or their national epidemiological situation.

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. The EURL team will participate twice to the *Brucella* international research conference (2016 and 2017), which will be held in the USA, in order to report to NRLs and DG-SANTE the main communications and results dedicated to *Brucella* diagnostic and epidemiology during the annual 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-SANTE.

3.3 Website management :

The website development aims at facilitating NRL access to information and exchanges between NRLs and the EURL. The website was opened in 2015 and different sections regarding EURL activities and brucellosis diagnosis have been developed. All NRLs have access to a EURL private section where overall information on brucellosis and the activities of the EURL are of public access.

In 2016 and 2017, the website development will be focused on scientific and pedagogical activities. The objective will be to develop a toolkit, including SOPs, technical and illustrated notes, principles of good laboratory practice and fact sheets. A series of fact sheets focusing on *Brucella* direct identification and serological diagnostic tools will be achieved to accompany some training sessions and to publish an educational booklet with sheets designed for the NRLs. In 2016, fact sheets will be dedicated to i) general *Brucella* properties , ii) bacteriological identification of *Brucella* ; in 2017, fact sheets will focus on two classical serological diagnostic tools : i) RBT, ii) CFT.

3.4 Working groups :

The EURL plans to moderate 1 or 2 electronic working groups within the programme, in order to improve information exchange between NRLs and/or to work on the development of common measures, standards and protocols. Topics will be selected in line with DG SANTE and NRLs major concerns during each annual workshop.

During the 2014 workshop, two topics were flagged as priority topics for *Brucella* diagnostic and control strategies : i) interpretation of MLVA patterns ; ii) strategies applied to confirm *Brucella* outbreaks in different epidemiological contexts. Both themes will be selected as first subject of discussion and exchange of views.

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
- One series of 4 fact sheets dedicated to *Brucella* diagnostic tools

- Information exchange between NRLs focused on of views regarding MLVA patterns and strategies applied to confirm *Brucella* outbreaks
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✓ **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 resources available on the website: articles, communications…
- Number of working groups moderated by EURL team in 2016-2017
- Number of scientists / technicians contributing to working groups

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

4.1 Establishment of *Brucella* specific guidelines for MLVA data interpretation for enhancing understanding of brucellosis spread

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.

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).

Previous *in vivo* and *in vitro* investigations (EURL 2013-2015 work programmes) evidenced the existence of some variable-number-tandem-repeat (VNTR) pattern variations between closely related *Brucella* strains, as previously reported in other pathogens, which may alter data interpretation. These findings highlight the need of determining *Brucella* specific guidelines for MLVA data interpretation.

EURL 2013-2015 work programmes have focused on the most relevant *Brucella* species in EU: *B. melitensis* and *B. suis* bv 2.

This task will continue in 2016-2017:

(i) to investigate the genetic stability of MLVA-16 markers in *B. abortus*

(ii) to analyse MLVA data obtained for *B. melitensis, B. suis* bv 2 and *B. abortus*

(iii) to establish *Brucella* specific guidelines for MLVA data interpretation at species and biovar levels

(iv) to assess these established interpretation criteria in well-identified epidemiological situations (*e.g.* same outbreak)

4.2 Improvement of molecular tests for a one-step identification of *Brucella* species and biovars by genomic approaches

Surveillance and control systems for a pathogen are based on a correct in-depth identification of the strains circulating in the study area, starting with correct taxonomy identification. Accordingly, to improve surveillance and control of brucellosis in Europe, extensive phenotypic and molecular characterizations are highly needed in different epidemiological contexts. In free areas, strain typing is essential in outbreak epidemiological investigations to identify the origin of introduction and prevent any re-emergence by targeting control/prevention measures. In infected areas, during the eradication phase, identification of circulating strains enables a better understanding of disease transmission between different species/reservoirs (included humans), thus providing some keys to improve the control strategy. During the control phase, in areas where mass vaccination is implemented, serological tools are reaching their limits to differentiate vaccinated and infected animals. In this context, vaccine strain characterization is required.

The phenotypic and biochemical characters guide the general identification of *Brucella* genus, as well as the identification at species- and biovar-levels. Furthermore, molecular identification is considered as an alternative or a complement to established phenotypic methods, which are time-consuming, fastidious and require strict biosafety facilities. The development of robust molecular tools offering a sufficient 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 (RT-PCR), which targets the genes *bcsp31*, IS*711* and *per*. Other multiplex PCR assays (AMOS, Bruce-ladder, Suis-ladder) are available for species- and/or biovar-identification of the most frequently isolated *Brucella* species in EU. But with a constantly upgraded taxonomy –introduction of at least 5 new species in 7 years–, these approaches are not exhaustive and could lead to a misidentification. Moreover, recent EURL findings from the 2013 "Bacteriology and Molecular

Biology" ILPT show challenges encountered by NRLs in a successful identification, especially regarding atypical strains, but also for the typical strains at the species level.

Fine Next Generation Sequencing (NGS) analysis, based on large-scale genome-wide data, will allow identifying novel markers including species-specific and biovar-specific molecular signatures among the *Brucella* genus. This will be applied to design new diagnostic/prognostic and phylogenetic tools.

To reach our objective, we propose to enhance *Brucella* genome database by Illumina and/or Ion Torrent technologies together with two assembly approaches (*de novo* and mapping), in order to identify respectively Single Nucleotide Polymorphisms (SNPs) and indels (Insertion/Deletion), as well as potential molecular markers to be applied for *Brucella* diagnosis:

1- Identification of key *Brucella* species and biovar-specific signatures: A representative panel of typical strains (reference and field isolates) of main species circulating in EU collected from domestic animals, wildlife and human, will be compared through genomic analysis. In addition, strains belonging to widespread species in EU, but harbouring an atypical phenotypic pattern (*i.e.* urease negative, oxidase negative, particular agglutination...) without reliable diagnosis will be in-depth investigated.

2- Identification of key *Brucella* vaccine-specific signatures: vaccine strains and field isolates will be compared to determine specific molecular key features for correct discrimination.

Selected biomarkers will allow discriminating at inter- and intraspecies levels, while being able to identify atypical strains. Once specific targets are identified, rapid, easy and reliable one step-species/biovar identification and genotyping tools will be developed.

Previous large-scale studies for RT-PCR validation directly from cattle, small ruminant and swine tissues have shown the limitations of direct detection techniques, especially in terms of sensitivity. Innovative technologies are currently available to detect and to genotype major pathogens as *Mycobacterium bovis* or *Bacillus anthracis*. So, high-throughput alternative approaches could be considered for *Brucella* diagnosis:

- The Luminex technology, based on the screening of a high region number with magnetic beads.
- The biochip technology which is an easy way to screen a large amount of targets in only one reaction in a short time.

Novel markers identified for species/biovar and vaccine/non vaccine strain discrimination, as well as for phylogeographic purposes will be used to design new more rapid and reliable brucellosis diagnosis tools and they will estimate prognosis/evolution of the disease through the determination and following-up of specific factors.

4.3 Main molecular mechanisms of long-term chronic brucellosis: investigation of biofilms and quorum sensing system

Brucella are facultative intracellular bacteria, capable of evading host defence mechanisms and of surviving inside the phagocytes for long periods. This intracellular persistence has been suggested as one explanation for the chronicity of brucellosis. The chronicity of *Brucella* infection in humans is well-known, and lesions most commonly reported are associated with focal disease. Although recognized in animals, *Brucella* persistence and its impact on animal health are less investigated than the human chronic disease.

Current serological tests are defined and validated for detection of acute infections, limiting the diagnosis of long-term chronic disease. Accordingly, chronic brucellosis represents a challenge for surveillance/eradication programs in the free countries, as well as in the endemic zones.
In France, the recent identification of a *B. melitensis* biovar 3 ibex reservoir –with spillover to domestic ruminants– emphasizes the silent persistence of *Brucella* in wildlife over a two-decade period. Genotyping studies, exhaustive surveillance systems, as well as wildlife behavioral studies strengthen the assumption that reactivation of a latent infection in an old animal might have contributed to the re-emergence of brucellosis. Similar conclusions might be hypothesized for the *B. abortus* bv 3 re-emergence in 2010 and 2012 in cattle in Belgium, a bovine brucellosis free country since 2003.

There is considerable evidence that some mechanisms for long-term non replicating persistence may be shared among chronic intracellular pathogens, as *Mycobacterium tuberculosis, Helicobacter pylori* or *Brucella* spp. In the microorganism world, 80 % of the microbial biomass resides in the form of biofilms, strongly associated with human and animal chronic infections. Indeed, biofilms are ubiquitous bacterial reservoirs involved in up to 80 % of all infections. Biofilms, able to attach on inert material as well as biotic surfaces (live tissues such as

epithelium cells), are functionally organized communities of aggregated cells embedded in a matrix of extracellular polymeric substances. Clinical observations emphasize the importance of biofilms (and their associated complications) to chronic and recurrent infections that resist host immune responses and biocides (antibiotics, disinfectants), highlighting ability of bacteria to persist *in vivo*.

In response to signal system –quorum sensing–, biofilm formation is well-documented, including in important veterinary pathogens, as *Staphylococcus epidermidis, Acinetobacter baumanii, Campylobacter jejuni*... The ability to form biofilms depends on specific structural genes and regulatory processes. Interestingly, this structurally and dynamically complex biological system is usually composed of several bacterial genera. It is assumed that strains unable to form biofilms can nevertheless be associated with bacterial communities (*e.g.*: *E. coli* O157:H7, *Listeria monocytogenes*).

The existence/importance of biofilms, neglected to date, in the overall process of brucellosis pathogenesis is unknown. Recent studies in genomics and genetics highpoint the putative ability of *B. melitensis* and *B. abortus* to form some biofilm-like structures, including exopolysaccharide production and clumping phenotype, in particular conditions (*e.g.* microaerobic conditions).

We propose (*i*) to determine in previously sequenced *Brucella* genomes the presence of necessary elements for biofilm formation and for quorum sensing system in Gram-negative bacteria and (*ii*) to develop *in vitro* models of *Brucella* biofilms in order to identify *Brucella* genes differentially expressed in comparison biofilm *vs* planktonic phase.

Better understanding of quorum sensing and detection of signal molecules produced for inter- and intraspecies communications, auto-inducers, within the biofilm would design new approaches to fight against *Brucella* persistence and to identify new biomarkers for diagnosis at early stages and follow-up of infections.

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

✓ **Expected outputs :**

- Determination of *Brucella* specific guidelines for MLVA data interpretation

- Determination of key *Brucella* species/biovar-specific signatures from typical and atypical strains
- Determination of key *Brucella* vaccine-specific signatures
- Determination of new molecular signatures as novel diagnosis targets for *Brucella* chronic infections
- Improvement of the current real time PCR tool for direct detection or Determination of novel diagnosis tools
- Development of *in vitro Brucella* biofilm models
- Improvement of surveillance in EU and better knowledge of *Brucella* genotypes circulating in Europe

✓ **Performance indicators**

- Number of *B. abortus* strains investigated by MLVA-16 for marker stability study
- Scientific communication about *Brucella* specific guidelines for MLVA data interpretation
- Number of *Brucella* strains investigated by NGS
- *In silico* comparative analysis of genetic regions putatively involved into biofilm formation and regulatory system in Gram-negative bacteria, and especially in *Brucella*