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Maisons-Alfort Animal Health Laboratory

Bacterial Zoonoses Unit

For the attention of the *in vitro* diagnostic industry producers of detection kits for animal health diagnosis

Maisons-Alfort, the 10 December 2021

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Subject: Call for expressions of interest for the development of immunological diagnostic kits for the detection of tuberculosis in swine

Dear Sir or Madam

This call for expressions of interest (AMI) is part of a process initiated by the National Tuberculosis Reference Laboratory of ANSES-Maisons-Alfort for the validation of new commercial kits that can be used in laboratories approved to carry out first-line analyses of tuberculosis in swine. The kits presented must allow a simple, rapid detection of the agent sought in samples of interest such as serum or blotting media.
A specification of the expected performance of the suidae tuberculosis detection kits is joint to this letter. This document also describes the different stages of the evaluation process of the kits submitted to the NRL. We would be grateful if you could show your interest and return your first intention proposals for this project by **4 February 2022**. Please send your response by e-mail to LNR.tuberculose@anses.fr

After this date, we will consider that you are not interested in this proposal.

Yours sincerely

María Laura Boschiroli

SPECIFICATIONS FOR SUBMITTING IMMUNOLOGICAL DIAGNOSTIC KITS FOR SWINE TUBERCULOSIS (ELISA Technique) FOR THE INITIAL COMPLIANCE CHECK

Maisons-Alfort Laboratory for Animal Health

Bacterial Zoonoses Unit (UZB)

National Reference Laboratory

Tuberculosis

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Purpose	Development of animal health diagnostic kits
Target	**Mycobacteria**
Method	ELISA
Matrix	Serum / Blotter

Version	**00**
Application date	

Validation

First/Last name	*Position*	*Date*	*Signature*
Maria-Laura BOSCHIROLI	**Manager of the NRL**	**12/04/2021**	**MLB**

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Foreword

Bovine tuberculosis (bTB) is a disease whose diagnosis is subject to official analyses (GUIDANCE NOTE, DGAL/SDSPA/N2010-8305, 08/11/2010) and for which the Bacterial Zoonoses Unit of the Maisons-Alfort Laboratory for Animal Health has been the National Reference Laboratory since 1999 (Ministerial Order of 29 December 2009, as amended, appointing national reference laboratories in the areas of veterinary public health and plant health).

Caused mainly by *Mycobacterium bovis*, it is a major infectious disease in the cattle sector and a very expensive health problem for farmers and the State. There has been a collective control and eradication campaign in force for this regulated Category 1 disease in France for over fifty years. As a result, France is recognised as free of bTB – less than 0.1% of herds affected – with this strategic status facilitating trade. However, although the EU's objective is to eradicate the disease across the continent, this goal – including in France – seems to be moving further away, largely because of the presence of *M. bovis* infection in many wildlife species in several regions where the disease seems to be becoming entrenched.

The NRL recommends and disseminates analytical screening methods for this disease in accordance with the DGAL's requirements. In this context, the screening analyses are regulated and the kits associated with these diagnostic methods must undergo an initial check and a batch compliance check by the NRL (Article R200-1 of the French Rural and Maritime Fishing Code).

Before new diagnostic kits can be submitted for the initial check by the NRL, the developer must prepare a validation file for tuberculosis screening by immuno-serological analyses.

Introduction

These specifications explain to applicants (producers and distributors of reagents) the general prerequisites for submitting an immunological diagnostic reagent (ELISA technique) for the NRL's initial compliance check, with a view to obtaining an initial certificate of compliance as provided for in Article R. 202-37 of the French Rural and Maritime Fishing Code.

They also describe the format and content of the technical file to be submitted by applicants, which defines, for each of the parameters specified by the NRL, the expected level of performance and the means to be used to assess it. The expected performance results are mainly based on technical capabilities and the needs according to the application objectives.

All data provided to the NRL by applicants are and will remain confidential.

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1. Reference standards and reference materials

Standards

- AFNOR XP U 47-310 standard, Animal health analysis methods – Verifying biological reagents for immunological techniques used in the area of animal health
- AFNOR NF U 47-301 standard, Animal health analysis methods – Submission file for verifying biological reagents used in the area of animal health
- AFNOR NF U 47-300 standard, Animal health analysis methods – Terminology
- AFNOR NF U 47-020 standard, Animal health analysis methods – Good practice guide for processing the sample submitted for immuno-serological analyses
- AFNOR NF U 47-019 standard, Animal health analysis methods – Good practice guide for the implementation of ELISA techniques
- OIE Terrestrial Manual "Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases"
- Vade-mecum on reagent checks at ANSES (web page address)
- Richomme C, Boadella M, Courcoul A, Durand B, Drapeau A, Corde Y, Hars J, Payne A, Fediaevsky A, Boschiroli ML. Exposure of wild boar to *Mycobacterium tuberculosis* complex in France since 2000 is consistent with the distribution of bTB outbreaks in cattle. PLoS One. 2013;8:e77842. (10.1371/journal.pone.0077842)
- Richomme C, Courcoul A, Moyen JL, Reveillaud É, Maestrini O, de Cruz K, Drapeau A, Boschiroli ML. Tuberculosis in the wild boar: Frequentist and Bayesian estimations of diagnostic test parameters when *Mycobacterium bovis* is present in wild boars but at low prevalence. PLoS One. 2019;14(9):e0222661. Published 2019 Sep 24. doi:10.1371/journal.pone.0222661
- Protocol for blotter preparation before analysis with the reagent (Annex A of these specifications)

Reference materials

There is currently no international standard serum available for swine tuberculosis. The reference laboratory has a freeze-dried in-house reference material (IRM) and other materials of varying status:

Reference material	Title	Test used
Negative pig serum	Negative SPF pig serum	Ingenasa INgezim TB porcine kit
Positive pig serum	Strongly positive	Ingenasa INgezim TB porcine kit
IRM blotter	Weakly positive	Ingenasa INgezim TB porcine kit
Freeze-dried IRM serum	Weakly positive	Ingenasa INgezim TB porcine kit

2. Definitions

The definition for each parameter is given in the introduction to the corresponding section. If not specified, the definitions for the terms used will be those in the above-mentioned reference standards.

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3. Background and reagent application objectives

Background

A national surveillance programme for tuberculosis in wildlife, Sylvatub, has been in place since late 2010. In addition, the Ministerial Order of 7 December 2016 on certain surveillance and control measures against tuberculosis when this disease is identified in wildlife (NOR: AGRG1635531A) has been in force since January 2017. This provides a regulatory framework for prefects' actions in the prevention, surveillance and control of tuberculosis when wildlife are infected, as well as in the regulation of populations, collection and destruction of viscera, intervention in hunting parks/penned areas, and the requirement to obtain the health status of released farmed animals.

Reagent application objectives

Since 2018, serological tests with the only commercial kit available to date (Ingenasa INgezim TB porcine) have been used. However, it is important that official analyses can rely on other commercial kits, hence the need to validate new ones. These official serological tests can be used in the above-mentioned programme for free wildlife, but also to screen for swine tuberculosis in regions such as Corsica where this disease is present in the pig sector.
The method needs to be transferable to the departmental veterinary laboratories in the network of official laboratories that was set up for carrying out first-line analyses (in progress).

The kits submitted must be capable of simple, rapid detection (within one day) of the agent screened for in samples of interest (sera or on blotters).

The results expected in these specifications must be in line with the defined objectives, which include:

- Contribute to demonstrating the absence of infection in a given population;
- Certify the absence of infection or the presence of the infectious agent in individual animals or in products for the purposes of trade/movement;
- Contribute to the eradication of a disease or the elimination of an infection in given populations;
- Confirm the diagnosis of clinical cases (including confirmation of positive screening tests);
- Estimate the prevalence of an infection or exposure to enable risk analysis;
- Determine the immune status of individual animals or populations.

4. Description of the reagent and the batch to be verified

Applicants should provide a precise description of their reagents: trade name and description, packaging, place(s) of manufacture, verification and packaging of the finished product, analytical principle, composition and conditions of use (protocols for use, types of samples, area(s) of use, precautions for use, etc.).
A precise description of the method and the raw materials critical to performance (biological components used, manufacturing processes, etc.) should also be provided, with the exception of data relating to industrial secrecy. The NRL is required to keep the information in the file confidential.
The storage conditions for the reagent and the validity period of the batch should be specified, as well as any information on the tests that enabled them to be established (in particular, the conditions and results of aging tests).
The batch submitted for inspection must be one that was manufactured and packaged under final marketing conditions and identified by a unique number. The batch number, size and validity period should be described, along with the batch number of the individual reagent components.

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Applicants should attach the following to the technical file:

- For each of the active ingredients (biological components) and each of the raw materials (chemical components), the description, manufacturer name and contact details, manufacturing process[1], packaging method (nature of the container, closure method, volume), storage conditions and safety data sheet,
- The draft instructions, **in French at least**, written according to the recommendations in Annex B of the NF U47-310 standard,
- The label templates for the reagent or each of the components,
- The internal quality control certificates of the batch submitted for verification.

5. Quality control (for category B reagents)

The supplier should describe the quality control procedures followed and the acceptance criteria for batch release.

6. Reagents to be provided by applicants for the compliance check

The manufacturer/supplier should provide, free of charge, at least one complete kit with at least 2000 reactions (to be specified with the NRL upon submission of the dossier). These reactions will enable the NRL to carry out an initial batch check and verify any other parameters it deems necessary (e.g. the method's limit of detection, intra-laboratory repeatability and reproducibility test, additional tests of diagnostic sensitivity and specificity, etc.).

If there is a regulatory requirement in the method for batch-by-batch reagent verification, the manufacturer/supplier undertakes to provide, free of charge, one reagent kit per manufactured batch at a later date. If batch-by-batch verification is not a regulatory requirement, the NRL may have to carry out spot checks during the validity periods of the kits and in this case the manufacturer/supplier also undertakes to provide, free of charge, the equipment needed for the verification tests.

7. Technical file to be submitted by applicants

Important note: The list and definitions of the parameters described below are based on the NF U47-310 standard. The data must be presented in the validation file according to the requirements defined by the NRL in these specifications. In particular, for each of the parameters, applicants must indicate the different types of samples used (reference, control, field), specifying how they were selected and characterised (origin, status, etc.). They should also specify the verification methodologies (protocols) used and include raw data on the results obtained.
A performance assessment must be carried out for each of the matrices for which the test is applicable and for each of the different technical protocols mentioned in the instructions.

Outsourcing: Some or all of the studies to characterise the reagents may be carried out in one or more external contract laboratories. The tests must then be performed by laboratories independent of the manufacturer and, as far as possible, accredited or approved for implementation of the diagnostic technique in question. Once validated by the head of the contract laboratory concerned, all raw results and, where applicable, results processed according to the manufacturer's instructions, must be reported to the NRL.

[1] with the exception of data relating to industrial secrecy

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7.1 Samples (biological matrices)

Applicants must describe the samples that can be analysed with the proposed reagent. In cases where more than one type of sample can be analysed, reagent performance should be assessed for each sample type.
Applicants should specify the procedures followed for preparing the samples and test specimen.

7.2 Validation of the test series and interpretation of the results

The conditions for validating the test series indicated in the instructions should be justified by suitable test results.
Interpretation of the results (criteria for test validity, calculation formula, etc.) and the threshold(s) of interpretation are left to the applicant's discretion. Applicants should describe the methodology followed (number and description of samples, calculations and statistics) and the results obtained to determine the threshold(s) according to the application objective(s).

7.3 Characterisation of serological reagents for qualitative techniques

Applicants should submit the tests carried out to determine the values of the assessed parameters and the results obtained, as described below:

Analytical specificity – exclusivity

Analytical specificity is the probability of the reagent obtaining a negative response for a sample not containing the target analyte.
Exclusivity is the ability of a reagent to specifically detect one target analyte while not detecting any of the other analytes that could potentially cause cross-reactions.
The manufacturer should specify the method used to determine the number of samples, the origin and nature of the samples, as well as the status of the animals. These samples must also be analysed by another recognised method.
Exclusivity testing should be performed on samples without antibodies to tuberculosis, including samples with or likely to have false positive serological reactions.
Cross-reactivity must be zero (100% specificity).
All results obtained must be submitted to the NRL.

Analytical sensitivity (or limit of detection)

The analytical sensitivity (or limit of detection) is the minimum amount of analyte that gives a positive response with the reagent in question. Analytical sensitivity is assessed by analysing the dilution of one or more reference materials, including the one that defines the required level of detection (RLOD). Around the "Corsica 3" serum dilution of 1:64.

Consistency of the dose-response relationship

The dose-response relationship should be verified on at least four dilutions using a strongly positive reference material able at certain dilution levels to cover the linearity area and the inflection points, and encompassing the RLOD.

Diagnostic sensitivity and specificity

"Diagnostic" sensitivity (DSe) is the proportion of a number of samples representative of the population corresponding to the areas and limitations of use specified in the instructions and defined as positive for the given target (e.g. infected or vaccinated; see the "Definitions" section of these specifications) that gives a positive result with the reagent submitted for testing according to the threshold(s) defined by applicants or the regulations in force.

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"Diagnostic" specificity (DSp) is the proportion of a number of samples representative of the population corresponding to the areas and limitations of use specified in the instructions and defined as negative for the given target (e.g. disease-free; see the "Definitions" section of these specifications) that gives a negative result with the reagent submitted for testing according to the threshold(s) defined by applicants or the regulations in force.
A confidence interval for the percentages should be determined, which is calculated according to the number of samples tested.
These criteria are estimated from a panel of field samples of known positive and negative status (determined by a bacteriological and/or PCR reference method and also with regard to the epidemiological bTB context at the regional level) that is representative of the region (country) where the test will be used. The number of samples should take account of the desired degree of confidence in their estimation, and their availability. The NRL recommends targeting a 95% confidence level, with a 5% or 2% error in estimating the DSe or DSp.
Different results are acceptable depending on the intended use of the test, but the NRL recommends a DSp of at least 96% and a DSe of at least 73%.

Intra-test repeatability

Repeatability is the closeness of agreement between repeated analyses of the same sample in the same laboratory during the same test (same reagent, same operating conditions).
A sample of a comparable level of detectability to the RLOD should be analysed on three full plates under repeatability conditions.
The coefficient of variation (CV) should be below 10%. The raw values of the three plates and the three CVs must be comparable.

Intra-laboratory reproducibility

This is the closeness of agreement between repeated analyses of the same sample with the same reagent in the same laboratory in several test series, varying the operating conditions of the same technical protocol.
Intra-laboratory reproducibility should be estimated from three dilution levels of the same sample, within the linear range, with a level of detectability comparable to the RLOD. These tests should be analysed in six different analysis series, varying the factors most likely to influence the precision of the test, and should be carried out over several days by at least two different operators.
The coefficient of variation (CV) should be below 20%.

Inter-laboratory reproducibility

This is the closeness of agreement between repeated analyses of the same sample in different laboratories with the same reagent and following the same technical protocol.
Where data allow (numerical data), the repeatability and reproducibility standard deviations, as well as the associated coefficients of variation, should be calculated on the transformed data (units, percentage of inhibition, for example).
Inter-laboratory reproducibility should be determined at least three times by at least three different laboratories (including the NRL) testing the same panel of samples with the following characteristics: blind analysis, a panel of at least 10 samples, comprising around 20% negative and 80% positive samples, and including three dilution levels of the same positive sample, within the linear range, with a level of detectability comparable to the RLOD.
The tests should be carried out using the same protocol, reagents and equipment.
The coefficient of variation (CV) should be below 20%.

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Robustness

This is the closeness of agreement between repeated analyses of the same sample with the same reagent in the same laboratory under the operating conditions and limitations specified in the technical protocol.
The robustness study therefore enables the protocol's conditions of use to be validated.
It should be assessed on the method's most critical parameters by testing in duplicate the RLOD or RLOD equivalent, a moderately positive sample, a strongly positive sample and a negative sample.

Robustness should be tested on three samples distributed across the linearity range and repeated in duplicate. The results should not differ significantly for each sample.
The following parameters should be varied:

- Incubations conditions (if mentioned in the instructions):
    - Temperature
    - Incubation time
- Washing type (manual/automatic)
- "Pause" before final reading

Stability

Evidence of the reagent's stability over time should be provided by applicants (depending on the method they choose, e.g. accelerated ageing).
The manufacturer should define the critical points that could affect reagent validity.
In cases where one or more reagent components may be used several times after opening or reconstitution, applicants should provide stability data on the storage times and conditions indicated in the instructions.
All stability studies should be carried out on at least two negative samples, two samples at the RLOD or RLOD equivalent and two positive samples, and must be performed over a period at least 10% longer than the storage time set by applicants. The principles regarding acceptability of variation in results for each type of sample should be defined at the start of the study.
If, at the time the file is submitted, the available data do not correspond to the storage time and conditions specified in the instructions or packaging (e.g. rapid ageing), applicants undertake to update them by monitoring the stability of at least three batches manufactured under the final production and marketing conditions, and to send the data to the NRL as they become available.

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ANNEX A: Preparation of swine BLOTTERS before the ELISA

1. **Cut out discs of about 5-6 mm diameter from the blotters and place them on a flat-bottomed plate.**
2. **Add 200 µL of the appropriate kit diluent (example: INGENASA INgezim TB porcine 11.TBP.K1) to each well containing blotters (*intermediate plate*).**
3. **Cover the plate with film and incubate for 16 to 20 hours in an oven at 21°C.**

Example of an ELISA

The ELISA should be performed according to the supplier's recommendations
Note that it is important to equilibrate all reagents to room temperature

1. **In the ELISA plate, distribute:**
   - 100 µL of positive controls in two wells and 100 µL of negative controls in two others (without dilution).
   - The NRL IRM diluted to 1:20: 1:10 pre-dilution in the intermediate plate (10 µL serum + 90 µL dilution buffer) then 95 µL dilution buffer in the ELISA plate and 5 µL of the previous dilution.
   - The samples: 50 µL of suitable diluent (example: Ingenasa) and add to it 50 µL of diluate (*1:2 dilution*).
2. **Cover the plate with film and incubate for 60 minutes at room temperature (18-25°C).**
3. **Wash the plate three times as described in the supplier's instructions.**
4. **Add 100 µL of ready-to-use conjugate to each well.**
5. **Cover the plate with film and incubate for 30 minutes at room temperature (18-25°C).**
6. **Wash the plate five times as described in the supplier's instructions.**
7. **Add 100 µL of substrate to each well.**
8. **Incubate for 10 minutes at room temperature (18-25°C).**
9. **Add 100 µL of stop solution to each well in the same order as the substrate was added.**
10. **Read at 450 nm on an ELISA reader.**

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