Brucellosis
Specifications for validation of Antigen for
Rose Bengal Test

1 Scope

The present document describes the requirements for validation (fitness for purpose) of antigen for Rose Bengal test (RBT) in brucellosis diagnostic.

The standardisation and validation are critical steps when a method is intended for routine diagnostic use in multiple laboratories. Therefore, customers can have confidence in the results produced by the test. All diagnostic assays should be validated for the species which they will be used for, according to the new OIE and EU principles and methods.

To validate a method, the manufacturer must submit a file to the National Reference Laboratory for Brucellosis (NRL) including a descriptive administrative part of the reagent(s) and a technical part. The evaluation criteria are described below (3. Criteria for Rose Bengal Antigen validation). The dossier will be reviewed by the NRL and a report will be returned to the manufacturer (Annex A).

2 Normative references

- Principles and methods for validation of diagnostic assays for infectious diseases, *In*: The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2021, Chapter 1.1.6., OIE, Paris. https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf

- Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products. Official Journal of the European Union 17.03.2017, L95/1-142. Text with EEA relevance. ELI: http://data.europa.eu/eli/reg/2017/625/oj

- Brucellosis (*Brucella abortus*, *B. melitensis* and *B. suis*) (infection with *B. abortus*, *B. melitensis* and *B. suis*) (version adopted in May 2016), In: The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2021, Chapter 3.1.4., OIE, Paris.

- NF U47-003. Animal health analysis methods - Detection of antibodies against Brucellosis by the Rose-Bengal Plate Test. AFNOR, April 2009.

3 Criteria for Rose Bengal Antigen validation

OIE chapter 1.1.6 recognises four stages in the assay validation pathway (Fig.1). These stages comprise the essential data to evaluate a new serological assay. The stages 1 and 2 are the minimum required for a Provisional Recognition (with a satisfactory result). Although, for a Full Recognition of assay validity stages 1 to 3 have to be satisfactorily completed.

The manufacturer must provide the assay name, the targeted agent and the description of the test method protocol, including the equipment used, calculations of results, data transformations and result interpretation (more details in Annex A). All data presented must be obtained using the final protocol provided.

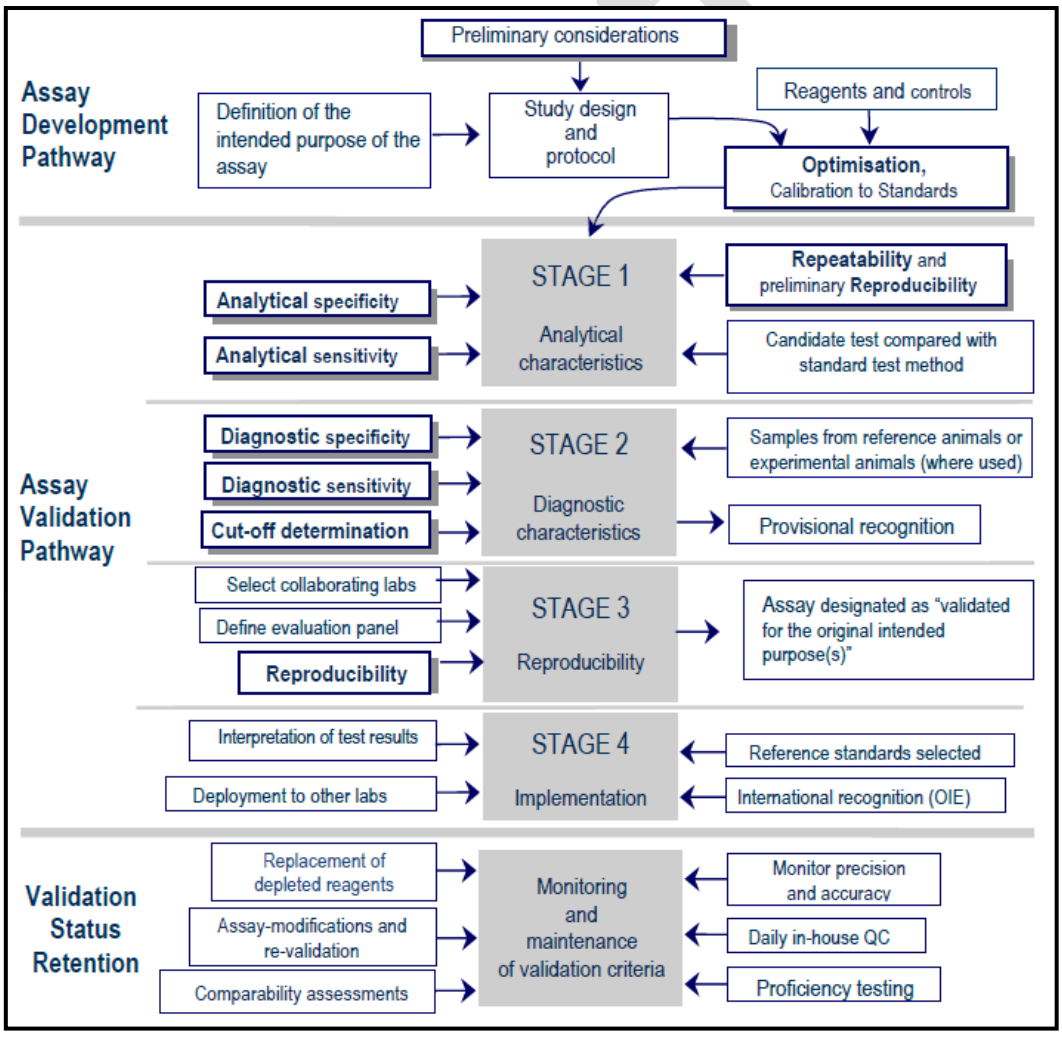

Figure 1. Principles and methods for validation of diagnostic assays for infectious diseases (source: OIE Terrestrial Manual, chapter 1.1.6)

3.1 Definition of the intended purpose(s) of the antigen

The first step is to define the intended purpose(s) of the antigen. The most common purposes are to:

I. Contribute to the demonstration of freedom from infection in a defined population.

   a. 'Free' with and/or without vaccination

   b. Re-establishment of freedom after outbreaks

II. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes.

III. Contribute to the eradication of disease or elimination of infection from defined populations.

IV. Confirmatory diagnosis of clinical cases (includes confirmation of positive screening test).

V. Estimate prevalence of infection or exposure to facilitate risk analysis.

VI. Determine immune status in individual animals or populations (post-vaccination).

3.2 Thresholds (cut-offs)

According to the OIE manual, serum tested with the RBT antigen for *B. abortus*, *B. melitensis*, and *B. suis* is considered positive with any visible agglutination.

3.3 Analytical sensitivity (Limit of detection)

Analytical sensitivity is determined by endpoint dilution of a positive sample in target matrix with replicate analyses of each dilution. The dilution series must extend to at least one dilution past end-point (negative/not detectable). The manufacturer must use one or more reference material(s) to define the Limit of Detection. It can be used an International Standard Serum or a Secondary or national standard

serum established against the above-mentioned OIE standard serum. This serum should be analysed at least three times (3-10 times).

The RBT should give a clearly positive reaction with 1/45 dilution, but not 1/55 dilution, of the Reference Serum diluted in 0.5% phenol saline or normal saline.

Data presentation: specify the reference sample, dilution series and result for each replicate.

3.4 Analytical specificity

Analytical specificity evaluates the differentiation of the target analyte from a range of other non-target but related analytes, such as cross-reacting antibodies derived from animals exposed to genetically related organisms or sera from animals with similar clinical presentations. The choice and sources of samples should reflect the test intended purpose (as indicated in 3.1).

The analytical specificity can be divided in:

I. Selectivity

   The selectivity control should be carried out (if possible) on representative samples containing antibodies directed against brucellosis (vaccinated and/or infected).

II. Exclusivity (cross-reactivity)

   The exclusivity control must be carried out on samples free of antibodies against brucellosis, including samples showing or likely to show false positive serological reactions.

   Cross reactivity (Analytical specificity less than 100%) may be acceptable depending on the intended use of the test.

Data presentation: the manufacturer must specify the determination of the number of samples, the origin and the nature of the samples, as well as the status of animals. These samples must also be analysed by another recognized method.

3.5 Diagnostic sensitivity (DSe) and specificity (DSp)

These criteria are estimated from a panel of samples from the field or from an experimental infection,

and of known positive and negative status (determined by a reference method) representative of the country or region where the test will be used.

Methods and statistical models to estimate DSe and DSp will depend on several factors including the availability or absence of existing reference (standard) test/s for comparative analyses, the identification of suitable negative populations and the availability of confirmed positive samples. For all intended purposes, the case definition for diseased populations must be clearly stated.

The number of samples must take into account the degree of confidence desired for their estimation and according to their availability. The EURL for Brucellosis suggests fixing a high degree of confidence at 95% with 5% or 2% error when estimating the DSe and DSp.

Data presentation: when results of the candidate assay are compared to a reference test, data should be presented in 2x2 tables with a confidence interval (ideally CI 95%).

3.6 Intra-assay repeatability

The repeatability of an assay provide data on the precision and accuracy of a test. A sample with a detectability level comparable to the LOD should be analysed 15 times under repeatability conditions (i.e. sample aliquoted into an appropriate number of individual containers and analysed at the same time).

Results must be equal for all samples (positive).

Data presentation: specify the reference sample, the sample dilution and results for each replicate.

3.7 Intra-laboratory reproducibility

Intra-laboratory reproducibility must be estimated from three dilution levels of the same sample, located in the linear range, for which a level of detectability is comparable to the LOD. To calculate intra laboratory reproducibility, these evaluations must be carried out in six different runs, over several days,

in different periods of the day, by at least two different operators.

Qualitative results must be equal for all samples.

Data presentation: specify the reference sample, the three dilution levels and results for each run.

3.8 Reproducibility

Inter-laboratory reproducibility must be carried out by at least 5 different laboratories with 4 separate repetitions in each. Each laboratory has to receive four times the same panel of samples in order to take into account the sera stability. The panel of samples has to contain following characteristics: blind analysis, panel of minimum 20 samples comprising approximately 25% negative samples and 75% positive samples, including 3 dilution levels of the same positive sample; located in the linear range, for which a detectability level is comparable to the LOD.

The tests are carried out with the same protocol, the same reagents and comparable equipment.

Qualitative results have to be concordant.

Data presentation: specify the panel samples (status, titre), the three dilution levels and results for each laboratory. Individual results for each laboratory and panel have to be presented.

3.11 Verification of stability

The stability of the finished product must be assessed for the period defined by the manufacturer under the recommended storage conditions.

The manufacturer must define the critical points which may influence the validity of the reagents.

Qualitative results have to be concordant.

Data presentation: specify the reference sample(s), the stability conditions and results for each the stability condition.

Annex A: Conformity Report for Rose Bengal Antigen Validation

This annex is an example of 'Conformity Report' for validation of Rose Bengal Antigen for brucellosis diagnosis.

Supplier / Manufacturer	
Commercial name	
Product reference	
Description	
Animal target	
Sampling matrix	
Operating Procedure	

EVALUATION CRITERIA	**RESULT** *(Passed / Need more data / Failed)*
Administrative file *(Name and address of the person responsible for placing the product on the market; accreditation or certification, name and address of manufacturer; accreditation or certification, trade name and denomination of the reagent, place(s) of manufacture and control, place(s) of packaging, number and size of the lot subject to control, date of the file signature of supplier's manager.)*	
Presentation of the reagent and reference materials *(Description of the reagent, technical protocol and formulation of the results, detailed instructions for use including the different production phases, reading methods and the critical stages of the reaction, precise rules for interpreting the results, reporting of known interferences, terms and duration of storage, security form, maximum number of reactions that can be carried out under the conditions of use provided for in the instructions.* *Reference material information: name, reference code, batch number, presentation, origin, dilution matrix, special preparation conditions, homogeneity.)*	
Label and notice *(It must be in the country's language and at least in a universal language, title, name of manufacturer, disease concerned, batch, storage/preservation conditions, volume, biosecurity conditions.)*	
Internal control analysis certificate *(Technical file)*	
Manufacturing process *(The critical steps for manufacturing or imposed by a document are presented in the form of a commented diagram. When the product may present a biological risk for the user or the environment, the conditions for inactivation of the pathogen and the methods for controlling this inactivation are described.* *Primary packaging: the nature of the containers and the closing methods as well as the minimum volume per packaging unit are specified.* *Identification: the process for identifying the batch of the antigen is described. In the case of further processing, the finished product is defined by a unique batch number for processing session.)*	
Definition of the intended purpose(s)	

Thresholds (cut-offs) *(Results must be as defined in the OIE manual, the manufacturer must describe the methodology followed and the results obtained.)*	
Analytical sensitivity (Limit of detection) *(Results must be as defined in the OIE manual, the manufacture must specify the reference sample, dilution series, result for each replicate.)*	
Analytical specificity *(The manufacturer must specify the determination of the number of samples, the origin and the nature of the samples, as well as the status of animals. These samples must also be analysed by another recognized method.)*	
Diagnostic sensitivity (DSe) and specificity (DSp) *(Results are expected according to the intended purpose(s) choice as described in 3.1:* *I and II. for screening test: high DSe (low false negative); for confirmatory tests: high DSp (low false positive)* *III. moderate to high DSe* *IV and VI. high DSp* *V. moderate DSe and DSp)*	
Intra-assay repeatability *(At least 15 repetitions, results by sample have been shown, qualitative results have to be concordant.)*	
Intra-laboratory reproducibility *(Three dilution levels of the same sample, located in the linear range, for which a level of detectability is comparable to the LOD must be tested in six different runs, over several days, in different periods of the day, by at least two different operators. Qualitative results have to be concordant.)*	
Reproducibility *(Qualitative results have to be concordant. Individual results for each laboratory and panel have to be presented.)*	
Verification of stability *(Qualitative results have to be concordant.)*	