Brucella typing

Standard Operating Procedure

1 Scope

The present document describes a standard technique that allows establishing species and biovars of an identified *Brucella* culture. Species identification is based on two main sets of properties: lysis by phages and oxidative metabolic profiles on selected amino acid and carbohydrate substrates. The described identification techniques enable to establish species and biovar of specific *Brucella* culture.

SAFETY PRECAUTIONS

The laboratory shall take all precautions in order to guarantee the necessary bio safety, for both the operator and the environment, against the biological and chemical hazards due to the activities conducted according to this document.

The operator may be infected with *Brucella* through skin or conjunctival contact, oral or respiratory mucosa with the aerosols containing *Brucella*. Laboratory biosafety measures aim at the retention of aerosols: all operations that may produce aerosols are to be performed in a biological safety cabinet, particularly while handling and culturing samples.

2 References

2.1 Normative references

- Brucellosis (Infection with *B. abortus, B. melitensis, B. suis*). *In*: The WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees), Chapter 3.1.4, WOAH, Paris, *Version adopted by the World Assembly of Delegates of the WOAH in May 2022.*
  https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.01.04_BRUCELLOSIS.pdf
- Ovine epididymitis (*B. ovis*), *In*: The WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees), Chapter 3.8.7, WOAH, Paris, *Version adopted by the World Assembly of Delegates of the OIE in May 2015.*
  https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.08.07_OVINE_EPID.pdf
- Quality management in Veterinary testing Laboratories. *In*: The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees), Chapter 1.1.5, OIE, Paris, *Version adopted by the World Assembly of Delegates of the OIE in May 2017.*
  http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/1.01.05_QUALITY_MANAGEMENT.pdf
- Alton G.G., Jones L.M., Angus R.D. & Verger J.M. (1988). Techniques for the Brucellosis Laboratory. Institut National de la Recherche Agronomique, Paris, France.
- 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
- ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories.

2.2 Regulatory references

Regulations concerning *Brucella* are strict and complex. The reader is invited to get in contact with corresponding official departments. See also the above mentioned OIE Manual reference for bio-safety precautions.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply:

3.1 *Brucella*

Brucella are gram-negative coccobacilli, non-motile, that form neither spores nor capsules. They neither ferment lactose on Mac Conkey agar, nor produce acid from glucose; they reduce nitrate; they are citrate-, indole- and VP-negative and they do not produce any haemolysis on blood agar. *Brucella* are catalase-positive and usually oxidase- and urease-positive. All smooth *Brucella* spp. strains show complete cross-reaction with each other, but not with non-smooth variants, in agglutination tests with unabsorbed polyclonal antisera. Cross-reactions between non-smooth strains can be demonstrated as well with unabsorbed anti-R sera. Lipopolysaccharide (LPS) comprises the major surface antigens of the corresponding colonial phase involved in agglutination. The S-LPS molecules carry the A and M antigens, which evidence different quantitative distribution among the smooth *Brucella* spp. strains.

3.2 CO_2-dependent strains

A CO_2-dependent strain presents a significantly more important growth when incubated for a specific time on a specific medium with a higher CO_2 level (5 % to 10 %) than in normal atmosphere.

4 Principle and reaction

After a culture of any suspected strain, its membership to the *Brucella* genus is established through "*Brucella* culture and genus identification" Standard Operating Procedure (SOP).

Species and biovar typing of the bacteria identified as belonging to the genus *Brucella*, is carried out on isolated colonies based on the following tests:

- Carbon dioxide (CO_2) requirement
- Hydrogen sulphide (H_2S) production
- Serum requirement
- Dye thionin and basic fuchsin sensitivity
- Lysis by Tb, Wb, Iz1 and/or R/C phage
- Growth in the presence of Penicillin, i-erythritol and/or streptomycin (for identification of vaccine strains S19, RB51 and Rev.1).

Once the typing is completed, the strain is stored in the strain library.

As an example, for classical *Brucella* species *B. melitensis*, *B. abortus* and *B. suis*, the identification at the biovar level is currently performed by four main tests described in the SOP "*Brucella* culture and genus identification": CO_2 requirement, production of hydrogen sulphide H_2S, thionin and basic fuchsin sensitivity, and agglutination with monospecific A and M antisera.

5 Diluents, culture media, reagents and other products

5.1 Diluents

5.1.1 Phosphate Buffered Saline (PBS)

Bacteria or samples to be ground should be suspended in Phosphate Buffered Saline (PBS). Solution of various compositions can be used as long as its' pH is neutral.

Example of composition:
NaCl.....8.5 g
Dipotassium phosphate (K_2HPO_4).....2.0 g
Monopotassium phosphate (KH_2PO_4) .....1.0 g
Distilled water (or of equivalent quality) .....up to 1,000 mL

Adjust pH to 6.8 ± 0.2; if the pH is too low, a 10 % solution of $NaCO_3$ can be added.

Sterilize by autoclaving (15 min, 121 ± 2°C).

This solution may be stored in bottles with stoppers for up to three months.

5.2 Culture media

5.2.1 Non-selective solid agar media

5.2.1.1 Non-enriched, non-selective solid agar media:

There is a large variety of dehydrated agar media that may be used for *Brucella* isolation or culture. The most frequently recommended are Tripcase or Trypticase-soy agar (TSA), blood agar base (BAB2), and Columbia agar. Other satisfactory media can be used such as serum-dextrose agar or glycerol-dextrose agar.

5.2.1.2 Enriched, non-selective solid agar media

Some *Brucella* strains (particularly *B. abortus* biovar 2) require the addition of serum in order to grow. Thus it is advisable to add 2 to 5 % (vol./vol.) of equine or bovine serum (5.3.3) to the media. For subcultures of strains that do not require serum enrichment, it is also advisable that media be enriched either with 2 to 5 % (vol./vol.) equine or bovine serum or with 0.1 % (weight/vol.) yeast extract (5.3.4) in order to enhance *Brucella* growth.

These media can be stored at 5 ± 3°C for up to three weeks.

5.2.1.3 Dye media

TSA and/or Serum-dextrose agar media are commonly used as a base media for dye test. When serum is added to medium containing dyes, the growth may be improved in comparison with growth without serum, even if the culture does not require serum.

Preparation of dye media:

- Prepare 0.1% stock solution of each dye (fuchsin and thionin) in distilled water.
- Heat the solutions in flowing steam for 20 minutes or in boiling water for 1 hour or filtering it with 0.22 µm filter.
- Stock the solutions of dyes in screw-capped bottles for maximum three month at 5 ± 3°C.
- To prepare the dye plates, add the appropriate quantity of stock solution to TSA and/or Serum-dextrose agar to adjust final concentration of dye at 20 µg/ml (1:50'000), mix well and dispense into plates while still hot. To increase the sensitivity of the test, the plates with dye concentration of 10 µg/ml (1:25'000) could be also added.

After the dye media have been poured into Petri plates and solidified, the plates may be placed in the incubator at 37°C overnight. If they are to be used on the day of preparation, the plates should be dried with the lid ajar and the medium facing downwards for 1-2 h in the incubator.

5.2.1.4 Antibiotic media

Enriched, non-selective solid agar media as TSA with addition of 2-5 % (vol./vol.) bovine or equine serum can be complemented with following antibiotics:

- Penicillin at 5 iu/ml
- i-erythritol at 1 mg/ml
- Streptomycin at 2.5 µg/ml
- Rifampicin at 250 µg/ml

Preparation of antibiotic media:

- Prepare a stock solution of each antibiotic in distilled water at the following concentration: penicillin at 10^4 iu/ml, i-erythritol at 1 g/ml, Streptomycin at 2.5 mg/ml and Rifampicin at 25 mg/ml.
- Filter the stock solution with 0.22 µm filter.
- To prepare the antibiotic plates, add the appropriate quantity of stock solution to TSA with addition of 2-5 % (vol./vol.) bovine or equine serum, mix well and dispense into plates while still hot. The final concentrations of antibiotics are: 5 iu/ml of Penicillin, 1 mg/ml of i-erythritol, 2.5 µg/ml of Streptomycin and 250 µg/ml of Rifampicin.

5.3 Other reagents

5.3.1 Control strain

Each laboratory must have at its disposal reference or well-biotyped *Brucella* strain.

Reference strains:

- *B. melitensis* biovar 1 strain 16M (ATCC 23456)
- *B. abortus* biovar 1 strain 544 (ATCC 23448)
- *B. suis* biovar 2 strain Thomsen (ATCC 23445)
- *B. ovis* 63/290 or *B. ovis* REO198

Vaccine strains:

- *B. abortus* S19 (BCCN R20)
- *B. abortus* RB51 (RB-51®)
- *B. melitensis* Rev. 1 (BCCN V4a)

5.3.2 Phage suspension

Following phage suspensions in trypcase soy broth (TSB) are used:

- Tbilisi (Tb) phage at routine test dilution (RTD) and at 10^4 x RTD
- Weybridge (Wb) phage at RTD
- Izatnagar (Iz1) phage at RTD
- R/C phage at RTD

The routine test dilution (RTD) is determined for each phage batch and reported on the product data sheet following manufacturer recommendations.

5.3.3 Sterile equine or bovine serum

Serum must be free from *Brucella* antibodies and passed through a 0.22 µm sterile filter. The serum is added at the rate of 2 to 5 % (vol./vol.) to the medium, which has been previously heated and then cooled to 56°C. Mix well and then, if applicable, add the selective mixture (5.2). Dispense into Petri dishes or in slants in ready-to-use capped tubes. This medium may be kept as indicated respectively in 5.2.

5.3.4 Sterile yeast extract

For strains that do not require serum enrichment, a previously sterilised (by filtration) yeast extract may be added to TSA-like media. Yeast extract is added at the concentration of 0.1 % (weight/vol.) to the basal medium, which has been previously heated and then cooled to 56°C. A selective mixture may be added (if applicable) at this point. Mix well and dispense into Petri dishes or in slants in ready-to-use capped tubes. The media prepared like this, may be kept as indicated respectively in 5.2.

5.4 Water

The chemical and bacteriological quality of the water used to prepare the different reagents will be verified. It must meet the requirements of the supplier and/or those imposed by the laboratory. These requirements shall enable a satisfactory implementation of the technique described in the present document. The water must always be sterile.

6 Equipment and plastic/glass ware

Conventional microbiology laboratory equipment and in particular:
- Temperature-controlled incubator set at 37 ± 2°C.

- Temperature-controlled CO_2 incubator set at 37 ± 2°C or any equipment able to produce and maintain an atmosphere containing 5-10 % CO_2, in a temperature-controlled incubator set at 37 ± 2°C.

- Temperature-controlled refrigerator at 5 ± 3°C.

- Temperature-controlled freezer at a temperature ≤ – 16°C.

- Containment and other biosafety equipment adapted and conforming to regulations in force (biosafety level 3), with at least one appropriate biosafety cabinet.

7 Operating Procedure

For each serie of tests, the control strains *B. melitensis* bv 1, *B. abortus* bv 1 and *B. suis* bv 2, mentioned above (5.3.1) are systematically tested in parallel and use as positive and/or negative control. The *B. ovis* strain and the vaccine strains are systematically added to characterise rough strain and vaccine strains, respectively.

Smooth (S) *Brucella* spp. cultures have a tendency to undergo transformation during growth, especially with subcultures, and dissociate to rough (R) forms. Verifying the colonial morphology of a culture is essential before performing any identification and typing test, since agglutination, phage lysis and dye susceptibility for growth may be altered with the R forms of naturally S *Brucella* strains. Accordingly, S-colony from S and intermediate (I) (level of dissociation < 5%) cultures, and R-colony from R and I (level of dissociation > 5%) cultures, were selected to use in the following tests. The phenotypic tests for R and/or I (level of dissociation > 5%) strains are the same for S *Brucella* strain, except for phage lysis (addition of the R/C phage) and monospecific sera (addition of the anti-R serum).

7.1 CO_2 requirement

The culture under examination should be tested for its CO_2 requirement immediately after isolation, before subculture of the original isolate has opportunity to develop independent CO_2 mutants.

7.1.1 Method

- A single colony of the bacterial culture under test as well as the reference strains is inoculated on two BAB2 tubes.

- Incubate one tube at 37 ± 2°C in a normal atmosphere and other tube at 37 ± 2°C in an atmosphere enriched with 5 to 10 % CO_2.

7.1.2 Reading and interpretation of results

The CO2 dependency should be read after 2-3 days of incubation.

- CO_2 independence (-): selective strain grows in both atmospheres (normal and CO_2 atmospheres);
- CO_2 dependence (+): selective strain only grows in CO_2 enriched atmosphere.

If a very few colonies grow in normal atmosphere, these should not be considered as real growth and the result will be CO_2 dependence.

7.2 H_2S production

7.2.1 Method

- A single colony of the bacterial culture under test as well as the reference strains is inoculated on single BAB2 tube.

- A strip of lead acetate paper is placed in the tube and is held between the plug and the side of the tube. Be careful, the paper must not touch the agar (the gap should be about 2 cm).

- Incubate the tube at 37 ± 2°C in an atmosphere enriched with 5 to 10% CO_2.

- The results of the H_2S production are read the next day, 18-30 h after incubation.

7.2.2 Reading and interpretation of results

- Production of H_2S (+): the strip of lead acetate paper is blackened from the lower part.
- No production of H_2S (-): the strip of lead acetate paper remains completely white.

Occasionally, a very thin black border may appear on the bottom of the strip of lead acetate paper for the negative control strain (*B. melitensis* bv 1 and/or *B. suis* bv 2). This type of result should be considered as negative. Instead, any other result showing blackening similar to the positive reference strain (*B. abortus* bv 1) should be considered as positive.

7.3 Serum requirement

7.3.1 Method

- A single colony of the bacterial culture under test as well as the reference strains is inoculated two non-enriched (5.2.1.1) and enriched (5.2.1.2) solid agar plates.

- Incubate one of each plates at 37 ± 2°C in a normal atmosphere and the remaining plates at 37 ± 2°C in an atmosphere enriched with 5 to 10 % CO_2.

7.3.2 Reading and interpretation of results

The serum requirement should be read after three days of incubation.

- Serum requirement (+): absence of the culture after 72h on the non-enriched plates indicates the serophilic character of the strain. In the same time, the culture has to be present on the enriched plates.
- No Serum requirement (-): presence of the culture after 72 h incubation on the both plates.

The culture should not be present on the plates incubated in enriched atmosphere for the strain presenting CO_2 dependence.

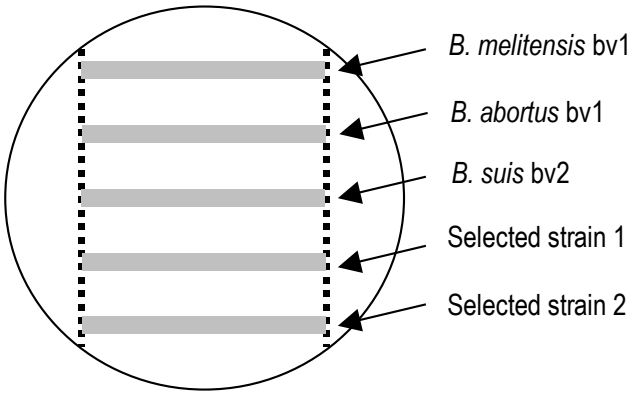
7.4 Growth in the presence of the dyes thionin and basic fuchsin

7.4.1 Method

- Prepare suspensions of the bacterial culture for testing as well as reference strains by suspending a loopful of recently grown culture (48-72 hours) in 1 ml of PBS. Take care to prepare suspensions at uniform turbidity.

- Label the dye plates with culture (under test and reference strains) number or code number. Remember, all strains should be tested in thionin and fuchsin concentrations at 20 µg/ml and 10 µg/ml (optional) as well as in normal and enriched CO_2 atmospheres.

- Immerse a sterile cotton swab on a stick in the bacterial suspension and, with the swab, make a single streak across each of the dye plates. Remember to streak each strain before on the 10 µg/ml plate (if added) and successively on the 20 µg/ml plate by rotating the swab to 1/4 between plates. Up to five suspensions can be inoculated in one single plate, as show in the following figure.



- Incubate half of the plates at 37 ± 2°C in a normal atmosphere and other half at 37 ± 2°C in an atmosphere enriched with 5 to 10% CO_2.

If there are more than two strains to be tested, the controls will not be re-tested under each concentration and atmosphere condition if the dye plates belong to the same production batch.

7.4.2 Reading and interpretation of results

The plates are observed after four to five days when cultures are sufficiently grown for the interpretation of the results.

- Growth in presence of dye (+): if the strain has grown.
- No growth in presence of dye (-): if the strain has not grown.

Only the result obtained under the atmosphere required by the strain is taken into account for typing.

7.5 Susceptibility to *Brucella* phage

The phages used in this test are described in the section 5.3.2. For *Brucella* R strains or I strains with a significant level of dissociation (7), the R/C phase at the RTD is also added.

7.5.1 Method

- Prepare suspensions of the bacterial culture for testing as well as reference strains by suspending a loopful of recently grown culture (48-72 hours) in 1 ml of PBS. Take care to prepare suspensions at uniform turbidity.

- Label the enriched, non-selective solid agar plates with culture (under test and reference strains) number or code number. Remember, all strains have to be tested with following phages: Tb at RTD and 10^4 x RTD, Wb at RTD and Iz at RTD. Moreover, for R strain or I (level of dissociation > 5%), the R/C phase at RTD is also added.

- Immerse a sterile cotton swab in the bacterial suspension and, make a single streak across each of the phage plates. Up to three suspensions can be inoculated in one single plate, as show in the figure below.

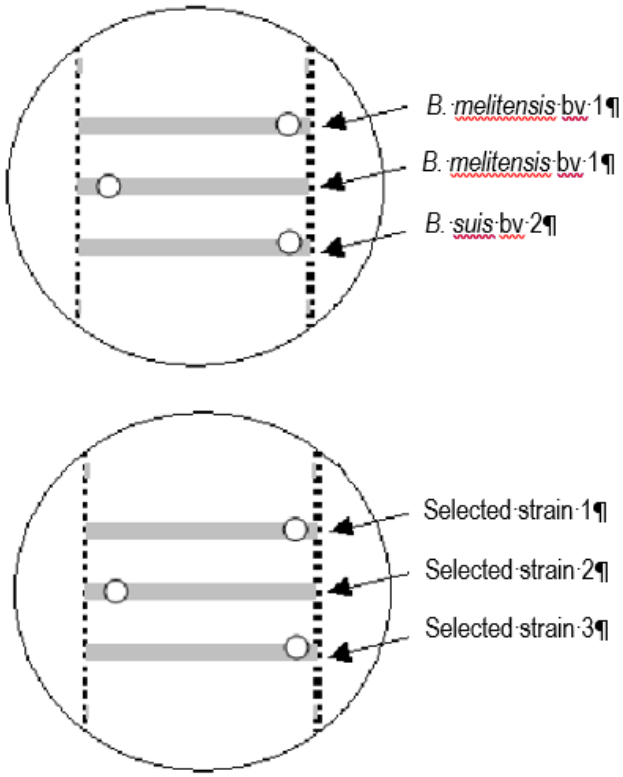
- Let the plates dry with the lids facing upwards before deposit the phage drop.

- In the phage plates, add one drop of the correct phage (Tb x RTD, Tb x 10^4 RTD, Wb x RTD and Iz x RTD) on each culture streak on the agar medium. To deposit the phage drop, use a Pasteur pipette or micropipette with very thin cone. Apply one drop (10-20 µL) of the phage on the culture streak starting from the left for the first strain, going down as shown in the figure below. Take care not to touch the agar with the pipette.

- Add also one drop of the R/C phage at RTD in the same way on each strake of the R/C plate.

- Let the page's drops dry before closing the plates.

- Incubate the plates at 37 ± 2°C in an atmosphere enriched with 5 to 10% CO_2.



7.5.2 Reading and interpretation of results

The plates are examined after 18-30 hours of incubation. Several results can observed:

- Phage lysis (+): complete absence of colonies where the phage drop was placed.
- Partial phage lysis (+ PL): partial culture growth where the phage drop was placed compared to the rest of streak or presence of lysis plaques in the drop area.
- Absence of phage lysis (-): presence of homogeneous culture growth where the phage drop was placed compared to the rest of streak.

For slow growing *Brucella* strain (ex. *B. melitensis* Rev. 1), the culture may be not sufficiently grown during the 18-30 hours of incubation. Then, the plates should be incubated for additional 24 hours before a new observation.

7.6 Growth in the presence of antibiotic

This complementary test will be carried out to characterize specifically *Brucella* vaccine strains.

7.6.1 Method

- Prepare suspensions of the bacterial culture under test as well as reference strains by suspending a loopful of recently grown culture (48-72 hours) in 1 ml of PBS. Take care to prepare suspensions at uniform turbidity.

- Label the antibiotic and control plates with culture (under testing and reference strains) number or code number following the scheme reported bellow.

- To characterise *B. abortus* S19 strain, the media used are:
  - TSA + 5% horse/bovine serum + i-erythritol (1 mg/ml).
  - TSA + 5% horse/bovine serum + Penicillin (5 iu/ml).
  - TSA + 5% horse/bovine serum (control plate).

- To characterise *B. melitensis* Rev.1 strain, the media used are:
  - TSA + 5% horse/bovine serum + Streptomycine (2.5 µg/mL)
  - TSA + 5% horse/bovine serum + Penicillin (5 iu/ml).
  - TSA + 5% horse/bovine serum (control plate).

- To characterise *B. abortus* RB51 strain, the media used are:
  - TSA + 5% horse/bovine serum + Rifampicin (250 µg/mL)
  - TSA + 5% horse/bovine serum (control plate).

The control strains used are the corresponding referent and its derivative vaccine strain (*B. melitensis* bv1 16M for *B. melitensis* Rev.1; *B. abortus* bv1 544 for *B. abortus* S19; *B. abortus* 2308 for *B. abortus* RB51).

- Immerse a sterile cotton swab on a stick in the bacterial suspension and, with the swab, make a single streak across each of the antibiotic plates. Take care not to use the same swab to streak the same culture on different antibiotic plates.

- Incubate the plates containing *B. melitensis* Rev.1 and *B. abortus* S19 at 37 ± 2°C in a normal atmosphere, while plates containing *B. abortus* RB51 at 37 ± 2°C in an atmosphere enriched with 5 to 10% CO_2.

7.6.2 Reading and interpretation of results

The plates are observed after four to six days, once cultures are sufficiently grown to interpret the results.

- Growth in presence of antibiotic (+): if the strain has grown on the antibiotic plate.
- No growth in presence of antibiotic (-): if the strain has not grown on the antibiotic plate.

Results can be validated only if the tested bacterial culture, as well as reference strains, grows on the control plate without antibiotic.

8 Storage and disposal of specimens, cultures and strains

Each laboratory should set up the provisions for the correct storage of samples until their disposal.

- Vaginal swabs must be kept at 5 ± 3°C and must be cultured without delay (if possible no more than 48 hours after sampling).

- Other specimens should be frozen at a temperature ≤ –16°C, if they cannot be processed without delay.

- In absence of contaminants, *Brucella* strains may be stored several weeks at 5 ± 3°C. For longer periods, cultures should be lyophilised or stored in a screw-capped tube at a temperatures ≤ –16°C, in tryptose broth with 15% (v/v) glycerol. For the maintenance of strains as well as for transportation to a reference laboratory, it is essential that only smooth colonies are selected.

Decontamination and disposal of samples, cultures and isolated strains must be performed in accordance to regulations in force.

Strains isolated and sent to a reference laboratory for confirmation of the *Brucella* genus and further specification and biotyping must be seeded on a non-selective solid medium and previously incubated 24 hours at 37± 2°C in an adequate atmosphere. Packaging and shipment must be performed in accordance to regulations in force.

9 Restitution of results

The results are based on the tables included in the OIE manual (2.1).

For laboratory's clients, restitution of the results is made as following:

A.2: Differential characteristics of species of the genus *Brucella*

A.3: Differentiation of biovars from species of the genus *Brucella*

A.4: Differential characteristics of *Brucella abortus* strain S19

A.5: Differential characteristics of *Brucella melitensis* strain Rev.1

Species and biovar (if applicable) determination of tested *Brucella* strains are confirmed only if their characteristics match with expected results as reported in the OIE manual (2.1). In the presence of atypical characters, i.e. different from reference strains, additional investigations will be required before species and biovar confirmation (epidemiological and molecular investigations).

10 Precision

The use of one or several reference strains (or a control strain that has been fully identified by a reference laboratory) during each series of tests allows for verification of the sensitivity and reproducibility of test conditions. The expected property of the reference or control strain must be found.

11 Analysis report

The analysis report must comply with the requirements of ISO/IEC 17025.