Brucella culture and genus identification

Standard Operating Procedure

1 SAFETY PRECAUTIONS

The laboratory shall take all precautions in order to guarantee the necessary 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 contact or contact of conjunctival, oral or respiratory mucosa with the aerosols containing *Brucella*. Laboratory biosafety measures are to 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 Scope

The present document describes a standard technique that allows research and bacteriological identification of the *Brucella* genus, in animal samples (ruminants, equidae, suidae, camelidae and carnivores, marine mammals, both wild and domestic). This is an isolation technique on solid culture agar medium. The described identification techniques enable a presumptive identification of bacteria of the *Brucella* genus.

3 References

3.1 Normative references

- Brucellosis (*B. abortus, B. melitensis, B. suis*). *In*: The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees), Chapter 3.1.4, OIE, Paris, *Version adopted by the World Assembly of Delegates of the OIE in May 2016.*
  http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/3.01.04_BRUCELLOSIS.pdf
- Ovine epididymitis (*B. ovis*), *In*: The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees), Chapter 3.7.7, OIE, Paris, *Version adopted by the World Assembly of Delegates of the OIE in May 2015.*
  http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/3.07.07_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.
- *Norme Française (French Standard)* NF U 47-105 - Méthodes d'analyse en santé animale, "Isolement et identification de *Brucella spp* autres que *B. ovis* et *B. canis* chez l'animal" avril 2004, *AFNOR, France.*
- *Norme Française (French standard)* NF U 47 109 – Méthodes d'analyse en santé animale, « Isolement et identification de *Brucella ovis* » décembre 2004, AFNOR, France.

3.2 Regulatory references

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

4 Terms and definitions

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

4.1 *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.

4.2 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.

5 Principle and reaction

Demonstration by Stamp (modified Ziehl-Neelsen) staining method of the presence of micro-organisms presenting the morphology of *Brucella* in animal samples is not specific and is thus considered as a suspicion of *Brucella*.

The bacteriological diagnosis is confirmed only by the isolation and the identification of *Brucella*. Direct isolation and culture of Brucella are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants. Sub-culture may be performed on non-selective media. However, the use of liquid media may be recommended for voluminous samples or for the purpose of enrichment.

Full identification of species and biovar (*i.e*: biotyping) is evidenced on an isolated strain through the association of cultural, morphological, biochemical, serological and phage-lysis characteristics *(Differential characteristics of species of the genus* Brucella *and differential characteristics of the biovars of* Brucella *species are listed in the OIE Manual Bovine Brucellosis chapter)*

Gram staining and presumptive research and identification specific techniques described in this document must be performed on each and every suspected colony before concluding a *Brucella* suspicion.

Naturally smooth (S) *Brucella* strains may naturally evolve into rough (R) strains, usually through subsequent subcultures, but sometimes at isolation. The serological, biochemical or phage-lysis characteristics of *Brucella* should be established from colonies that have been verified as being perfectly smooth, since this conditions the species and biovar identification by a reference laboratory.

6 Diluents, culture media, reagents and other products

6.1 Diluents

6.2 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.

6.3 Culture media

Culture media used for isolation (Petri dishes) or for subculture (Petri dishes or slants in tubes) are identical. When research is performed on samples that may be contaminated by extraneous organisms, media may be made selective by adding antibiotics or antifungals (6.4). The use of liquid media may be recommended for voluminous samples or for the purpose of enrichment. A wide range of commercial dehydrated basal media is available, e.g. Brucella medium base, tryptose (or trypticase)–soy agar (TSA).

6.3.1 Non-selective solid agar media

6.3.1.1 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 (BAB n°2), and Columbia agar. Other satisfactory media can be used such as serum-dextrose agar or glycerol-dextrose agar.

6.3.1.2 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 (6.6.2) 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.) of equine or bovine serum or with 0.1 % (weight/vol.) of yeast extract (6.6.3) in order to enhance *Brucella* growth.

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

6.4 Selective solid agar media

6.4.1 Farrell's selective solid agar medium

Take one litre of non-selective solid agar medium (6.3.1.1) previously heated up and cooled to 56°C (molten medium). Add the serum (6.6.2) and the following selective agents:

Polymixin B sulphate ..... 5 000 Units (5 mg)
Bacitracin..... 25 000 Units (25mg)
Natamycin.....50 mg
Nalidixic acid.....5 mg
Nystatin.....100 000 Units

Vancomycin .....20 mg

Follow the supplier's instructions to reconstitute (aseptically in sterile diluent) and use the antibiotics and antifungals.

Mix thoroughly before pouring directly into Petri dishes, cover with aluminium paper or sealed plastic wraps and keep at 5 ± 3°C up to eight days.

6.4.2 Modified Thayer-Martin's selective solid agar medium

6.4.2.1 GC Medium Base (2X)

Example:
Proteose Peptone No. 3 .....15.0 g
Corn starch .....1.0 g
Dipotassium phosphate (K_2HPO_4).....4.0 g
Monopotassium phosphate (KH_2PO_4) .....1.0 g
Sodium chloride.....5.0 g
Agar .....10.0 g
Water (5.4.1).....up to 500 mL
pH = 7,2 ± 0,2

Follow the supplier's instructions to reconstitute the medium and sterilise at 121 ± 2°C for 20 min.

Mix thoroughly before dispensing into appropriate containers with stoppers.

Cool to 56°C.

6.4.2.2 Haemoglobin solution (20 g/L)

Dissolve 10 g of haemoglobin powder for each L of medium desired in 1/2 volume of cold purified water.

For example:
Haemoglobin (freeze-dried powder).....10.0 g
Water (5.4.1).....up to 500 mL

Autoclave the solution at 121 ± 2°C for 15 min;

Mix thoroughly before dispensing into appropriate containers with stoppers;

Cool to 50°C.

6.4.2.3 GC Medium supplemented with haemoglobin (10 g/L)

Combine aseptically the haemoglobin solution (20 g/L) with 1/2 volume of autoclaved GC agar medium base 2X (5.2.2.1)

For example:
G.C. Medium Base (2X) .....500 mL
Haemoglobin solution (20 g/L) .....500 mL

The medium is added to the haemoglobin solution, not the other way around.

6.4.2.4 Modified Thayer-Martin's selective solid agar medium

Take one litre of GC Medium supplemented with haemoglobin (10 g/L) previously heated up and cooled to 50°C (molten medium). Add the following selective agents:

Vancomycin .....3.0 mg
Colistin methane sulphonate .....7.5 mg
Nystatin..... 100,000 UI
Amphotericin B .....2.5 mg
Nitrofurantoin .....10.0 mg

Follow the supplier's instructions to reconstitute (aseptically in sterile diluent) and use the antibiotics and antifungals.

Mix thoroughly before pouring directly into Petri dishes, cover with aluminium paper or sealed plastic wraps and keep at 5 ± 3°C up to eight days.

6.5 Selective CITA medium

Take one litre of blood agar base as basal component, supplemented with 5 % of sterile calf serum. Add the following selective agents:

Vancomycin ..... 20.0 mg
Colistin methane sulphonate ..... 7.5 mg
Nystatin ..... 100,000 UI
Amphotericin B ..... 4 mg
Nitrofurantoin ..... 10.0 mg

This antibiotic mixture can be prepared as follows: weigh vancomycin, colistin and nystatin in the same 50 ml sterile container, then rehydrate the mixture with 10 ml of a 1:1 solution of absolute methanol in sterile purified water. Weigh then nitrofurantoin in a sterile tube and dissolve it with 1 ml of 0.1 M NaOH solution (sterilised previously by filtration through a 0.22 µm filter). Finally, weigh 10 mg of amphotericin B in a 20 ml sterile container and dissolve with 1 ml dimethyl sulphoxide. Once fully dissolved (5–10 minutes are required), add 9 ml of 10 mM sterile phosphate-buffered saline (PBS) (pH=7.2 ± 0.2). The final concentration of amphotericin B would be 1 mg/ml; a total of 4 ml of this solution are required for 1 litre of medium. The remaining Amphotericin B suspension can be kept at 5°C ± 3°C for several days for further uses.

6.6 Other reagents

6.6.1 Control strains

Each laboratory must have at its disposal reference or well-biotyped *Brucella* strains (*B. abortus* biovar 1, *B. melitensis* biovar 1, *B. suis* biovar 2, *B. ovis*, for instance).

6.6.2 Sterile equine or bovine serum

Serum must be free from *Brucella* antibodies and passed through a sterilising 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 (6.4). Dispense into Petri dishes or in slants in ready-to-use capped tubes. The medium thus prepared may be kept as indicated respectively in 6.3.

6.6.3 Sterile yeast extract

For strains that do not require serum enrichment, a previously sterilised (through filtration) yeast extract may be added to TSA-like media. Yeast extract is added at the rate 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 thus prepared may be kept as indicated respectively in 6.3.

6.6.4 Crystal violet stock solution (observation of the colony morphology by the White & Wilson method)

Solution A: Crystal violet ..... 2 g
Absolute ethanol ..... up to 20 mL
Solution B: Ammonium oxalate ..... 0.8 g
Water (5.4.1) ..... up to 80 mL

Mix both solutions (A and B). The stock solution thus obtained may be stored in stoppered bottles at 5 ± 3°C for up to 3 months.

6.6.5 Solution for the oxidase test

Saturated solution of N,N-dimethyl-p-phenylenediamine (hemioxalate salt) in distilled water (or equivalent) to prepare fresh daily.

6.6.6 Christensen's medium (for the urease test)

Christensen medium available commercially is to be prepared according to suppliers instructions.
It may also be prepared from scratch as follows:

6.6.6.1 Basal medium

Composition:
Peptone .....1.0 g
Sodium chloride.....5.0 g
Monopotassium phosphate (KH_2PO4) .....2.0 g
Phenol red .....0.012 g
Dextrose .....1.0 g
Agar .....20.0 g
Water (5.4.1).....up to 1,000 mL

Preparation:

- Dissolve in a little cold water all products except the phenol red. Add up the rest of the water (6.7) boiling. Stir well;
- Check that pH is 6.8 ± 0.2 at 25°C;
- Add up phenol red;
- Dispense in 5 mL bottles;
- Sterilize at 121 ± 0.2°C for 15 min;
- Store in stoppered bottles at 5 ± 3°C for up to three months;
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6.6.6.2 Urea solution

- Prepare a urea solution at the rate of 20 % (weight/vol.) in water (6.7). Filtrate to sterilise (0.22 µm). This solution may be kept in stoppered bottles at 5 ± 3°C for up to six months;
- When necessary, melt the basal medium (6.6.6.1) then cool to 56°C. Add 5 mL of the urea solution, mix well and dispose into Petri dishes (3 Petri dishes per bottle or glass tubes in slope);
- The Petri dishes containing the medium are to be covered with aluminium paper or sealed plastic wraps and may be kept at 5 ± 3°C up to three weeks.
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6.6.7 Reagents for the slide agglutination of colonies

6.6.7.1 Unabsorbed hyper-immune anti-S-Brucella polyclonal serum

This is a serum capable to agglutinate S-*Brucella* colonies recently isolated. This reagent may be a serum from field cases in any animal species with a high titre of anti-S-*Brucella* antibodies or may be prepared in rabbits with S-*Brucella* cultures. It may be prepared in the laboratory or acquired commercially.

The neat serum is stored frozen at a temperature < -16°C in small aliquots and the dilute serum may be preserved by the addition of 0.01 % thiomersal or 0.5 % phenol saline and stored at 5 ± 3°C.

6.6.7.2 Unabsorbed hyper-immune anti-R-Brucella polyclonal serum

This is a serum capable to agglutinate R-*Brucella* colonies. This reagent may be a high titre serum from field cases with a high titre of anti-R-*Brucella* antibodies (*e.g. B. ovis* infection in rams) or may be prepared in rabbits with *B. ovis* cultures. This standard reagent can be obtained from the EU Reference Laboratory for Brucellosis (EURL) or can be prepared in the laboratory, provided that the activity of the reagent has been checked on a panel of R strains (*B. ovis*, *B. canis* and S-*Brucella* in the rough phase).

The neat serum is stored frozen at a temperature < -16°C in small aliquots and the dilute serum may be preserved by the addition of 0.01 % thiomersal or 0.5 % phenol saline and stored at 5 ± 3°C.

6.6.7.3 Anti-A and anti–M monospecific sera

These sera are available from the EURL or can be prepared in the laboratory provided that the activity of the reagent has been checked on a panel of *Brucella* strains, including *Brucella* species and biovars for which the agglutination with monospecific sera is essential for typing.

These sera may replace the above mentioned ones (6.6.7.1 and 6.6.7.2).

These sera are to be stored according to the supplier's instructions.

6.6.7.4 Negative control serum

Serum free from anti-S- and anti-R-*Brucella* antibodies.

The neat serum is stored frozen at a temperature < -16°C in small aliquots and the dilute serum may be preserved by the addition of 0.01 % thiomersal or 0.5 % phenol saline and stored at 5 ± 3°C. This standard reagent can be obtained from the EURL or can be prepared in the laboratory provided that the activity of the reagent has been checked on a panel of *Brucella* strains, including *Brucella* species and biovars for which the agglutination with monospecific sera is essential for typing.

6.7 Water

The chemical and bacteriological quality of the water used to prepare the different reagents shall 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.

7 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.

- Water bath (circulating water bath if possible).

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

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

- Centrifuge, refrigerated if possible, allowing adequate acceleration and equipped with devices preventing aerosols (*e.g*. safety buckets or containment rotors).

- Device for grinding and macerating tissues (*e.g.* Stomacher).

- Stereoscopic microscope with, if possible, obliquely reflected light.

- Microscope equipped with an oil-immersion objective and adequate magnification power (ocular 8x, objective 100x/1.25 if possible).

- Containment and other biosafety equipment adapted and conforming to regulations in force (biosafety levels 2 or, preferably, level 3), with at least one appropriate biosafety cabinet.

8 Sampling

All specimens must be conditioned according to standards or regulations in force, without preservative, in hermetically sealed containers. They must be transported without delay to the laboratory with temperature kept at 5 ± 3°C, according to regulations in force. With the exception of vaginal swabs which must be kept at 5 ± 3°C, all specimens that could not be delivered to the laboratory within 24 hours should be frozen at a temperature ≤ –16°C.

Upon reception at the laboratory, if the specimen cannot be processed without delay, it should be frozen at a temperature ≤ –16°C, excepted vaginal swabs which must be kept at 5 ± 3°C and must be cultured without delay (if possible no more than 48 hours after sampling).

The sensitivity of the *Brucella* culture diagnosis is relatively low and *Brucella* are often present in very small numbers in specimens. It is therefore recommended to culture several specimens to increase the chances of isolating the organism.

In order to optimise the sensitivity of the culture, the most valuable specimens are the following:

8.1 From live animals

- Females:
  - a swab of the uterus cervix (ecto- and endo-cervical areas) or, if not possible, a vaginal swab sampled in the 2-3 weeks following parturition or abortion;
  - a 10 mL milk sample from all quarters.

If a swab cannot be obtained, genital secretions are sampled. It is also possible to sample the foetal membranes and the aborted foetus or stillborn product (for cultural examination of the stomach content, lung and spleen). *These samples may contain enormous numbers of Brucella and must be handled, packed and transported with special care*.

- Males: semen;
- Both sexes: arthritis (synovial) or hygroma liquids.

8.2 From carcasses

- Tissues of the reticulo-endothelial (mononuclear phagocyte) system (lymph nodes, spleen, liver) in both sexes;
- The preferred lymph nodes (3 pairs when possible) are those of the head (parotid, retropharyngeal, mandibular, prescapular) and of the genitalia (medial iliac, inguinal) in both sexes and mammary lymph nodes in females;
- Pregnant uterus and udder in females (*samples difficult and dangerous to handle*);
- Testes and epididymes in males.

9 Specimen preparation for culture

The preparation of the sample shall comply with the requirements of the OIE Manual.

9.1 Cervix or vaginal swabs

The swab is withdrawn aseptically from the protecting tube or envelope and rehydrated in PBS if necessary (6.2).

9.2 Female genital secretions or semen

These specimens do not need any preparation.

9.3 Milk

Milk is centrifuged at 500-1000 *g* (*ca*. 5000 à 10 000 $m.s^{-2}$) for 15 min. Cream and pellet are sampled.

9.4 Tissues

Tissues must be macerated and ground as completely as possible (6.7), if necessary in presence of PBS (6.2.) in a proportion of 1/2 to 1/5 in order to get a suspension of adequate density for culture. Tissues may be previously cut in small pieces using sterile scissors and forceps in order to obtain an homogenous suspension more rapidly after grinding. If an important contamination of the sample is suspected, it is recommended to sample the internal part of the specimen.

9.5 Arthritis (synovial) or hygroma fluids

These specimens do not need any preparation.

9.6 Urine

Urine is centrifuged at 500-1000 *g* (*ca*. 5000 à 10 000 $m.s^{-2}$) for 15 min. The pellet is sampled.

10 Operating procedure

In each series of cultures and pre-identification of *Brucella*, control strains should be used to check the quality of media, reagents and culture conditions for the growth of *Brucella*. For the identification tests, it is recommended to use both a strain positive and a strain negative in each corresponding test.

10.1 Culture of specimens

10.1.1 Inoculation

The sensitivity of the *Brucella* culture diagnosis is relatively low and *Brucella* are often present in very small numbers in specimens. It is therefore recommended to inoculate several dishes of solid medium (6.3) to increase the chances of isolating the organism.

Direct isolation and culture of Brucella are usually performed on solid media. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants. However, the use of liquid media may be recommended for voluminous samples or for the purpose of enrichment. The sensitivity of culture increases significantly by increasing number of plates and the simultaneous use of different media (as an example, Farrell and CITA), due to inhibitory activity of nalidixic acid and/or Bacitracin on some *B. abortus* or *B. melitensis* strains.

Some *Brucella* strains are CO_2-dependent for growth. Therefore, each specimen should be inoculated on at least four plates, half being incubated in a normal atmosphere, half being incubated in an atmosphere enriched with 5-10 % CO_2.

Non-selective solid basal media (6.3) can be used (alone or in addition to the selective medium) provided that the specimen is not contaminated by extraneous organisms (*e.g*. in the case of semen or arthritis fluids sampled aseptically) or when *Brucella suis* biovar 2 or other *Brucella* sensitive to the Farrell's medium are expected.

The swab, rehydrated with PBS if necessary, is rubbed directly over the surface of solid selective medium (6.4). This must be performed as rapidly as possible after the sampling.

10.1.2 Female genital secretions or semen

Female genital secretions or semen are pipetted then spread directly on solid selective medium (6.4) with a swab-stick or a plastic spreader (*ca.* 0.2 mL per dish).

10.1.3 Milk

The cream and pellet obtained after centrifugation are pipetted then spread directly on solid selective medium (6.4) with a swab-stick or a plastic spreader (*ca.* 0.2 mL per dish).

10.1.4 Tissues

Pre-macerated and ground tissues, eventually suspended in PBS, are pipetted then spread directly on solid selective medium (6.4) with a swab-stick or a plastic spreader (*ca.* 0.2 mL per dish).

10.1.5 Foetal stomach content

Foetal stomach content are pipetted then spread directly on solid selective medium (6.4) with a swab-stick or a plastic spreader (0.1-0.2 mL per dish).

10.1.6 Arthritis (synovial) or hygroma fluids

Arthritis (synovial) or hygroma fluids are pipetted then spread directly on solid selective medium (6.4) with a swab-stick or a plastic spreader (0.1-0.2 mL per dish).

10.1.7 Urine

The pellet obtained after centrifugation is pipetted then spread directly on solid selective medium (6.4) with a swab-stick or a plastic spreader (0.1-0.2 mL per dish).

10.2 Incubation and reading

For each specimen, plates are incubated at 37 ± 2°C, half in a normal atmosphere, the other half in an atmosphere with 5-10 % CO_2.

Brucella colonies generally become visible on solid media after the cultures have been incubated for at least 3 days, sometimes later on selective media. After that time, it is advisable to routinely examine the cultures carefully, at least up to the 10th day of culture, so that any suspected colony can be sub-cultured before overgrown by contaminants. This must be done before cultures be discarded as negative.

After 4 days' incubation, *Brucella* colonies are round, usually 1–2 mm in diameter, with smooth margins. They are translucent and pale honey colour when plates are viewed in the daylight through a transparent medium. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker (for Farrell's medium).

As regards the examination on Thayer Martin's medium, *Brucella* colonies are not haemolytic, circular, convex, have unbroken edges, and are always of the rough type when examined by oblique illumination.

It is also advisable to consider the CO_2-dependence of the culture at that stage. If the strain is CO_2-dependent, further sub-cultures must be performed in a CO_2-enriched atmosphere.

Suspected *Brucella* colonies are picked and streaked on basal medium. If they are close to colonies of contaminants, they'd better be re-streaked on selective medium. In any case, the plate is streaked in such a manner that colonies are close together in some areas and separated in others.

If several types of suspected colonies are observed, representative colonies of each type should be picked and identified.

Morphology of the suspected colonies is immediately checked by Gram staining.

10.3 Validation and presumptive identification

10.3.1 Colonial morphology (S or R)

After incubation of 4-5 days, the plate is examined for colonial morphology.

Smooth *Brucella* spp. cultures have a tendency to undergo variation during growth, especially with subcultures, and dissociate to R forms. Verifying the colonial morphology of a culture is essential before performing any identification and biotyping test, since agglutination, phage lysis and dye susceptibility for growth may be altered with the R forms of naturally S *Brucella* strains. The two following techniques may be used.

10.3.2 Direct observation of colonies (Henry's method)

This technique requires a device allowing the examination of colonies through a stereoscopic microscope by obliquely reflected light.

- S colonies are round, glistening and blue to blue-green in colour;
- R colonies are less transparent, with a dry, granular appearance, and range in colour from matt white to yellow or brown.

10.3.3 Observation of colonies with crystal violet

- Dilute the crystal violet stock solution (6.6.4) 1/40 in sterile water (6.7) just before use;
- Flood the plates to be examined with the dilute solution for 15-20 seconds. Then the stain is removed and transferred into an autoclavable biological waste container;
- Observe the colonies, preferably through a magnifying glass or a stereoscopic microscope. Smooth colonies do not take up the dye. Rough or dissociated colonies are stained with various shades of purple and the surface may show radial cracks;
- Pick and streak rapidly the S colonies on a basal solid medium since stain may be toxic for colonies after a few minutes.

10.3.4 Agglutination test

- Place a microscope slide in a Petri dish;
- Take a small quantity of a suspected *Brucella* colony and suspend in a drop of anti-S-*Brucella* polyclonal serum or monospecific sera (6.6.7);
- Do the same with the negative control serum (6.6.7.4);
- Mix the sera with the culture, and within 5 minutes, examine for agglutination.

S-*Brucella* agglutinate the anti-S-*Brucella* polyclonal serum or the anti-A and/or M-monospecific sera. They should not agglutinate the negative control serum. If no agglutination is obtained with the abovementioned sera, or if the colony is rough or dissociated, do the same with the anti-R-*Brucella* polyclonal serum. This serum should agglutinate the dissociated or R colonies. These colonies usually do not agglutinate the negative control serum, however a slight agglutination may be observed.

Known R and S *Brucella* cultures should always be tested at the same time for comparison of speed and degree of agglutination.

10.3.5 Oxidase test

- Place a strip of filter paper impregnated with the saturated solution of N,N-dimethyl-p-phenylenediamine (hemioxalate salt) prepared fresh daily (6.6.5) in a Petri dish.
- Pick a portion of freshly grown colony and rub it on the strip.
- If the reaction is positive, the bacteria in contact with the moist paper turn dark pink within 10 s.

Brucella are usually “oxidase positive”, except *B. ovis, B. neotomae* and some African strains of *B. abortus*.

10.3.6 Urease test

- Use the ready-to-use Christensen medium or equivalent;
- Pick a portion of a freshly grown colony and seed it onto the medium.
- Let the plate at room temperature and observe it at regular intervals, up to 24 hours if necessary:
    - if the strain is “urease positive”, the colour of the medium changes from yellow to purple-pink almost immediately or in several minutes or hours.
    - if the strain is “urease negative”, the colour of the medium remains unchanged.

Brucella are usually “urease positive”, except *B. ovis* and the reference strain 544 of *B. abortus* biovar 1 as well as some *B. abortus* field isolates.

10.4 Interpretation

A strain may be considered as a probable strain of *Brucella*[1] when:

- its growth is possible on the Farrell’s medium;
- the isolated colony(ies) can be observed only after several days of culture with an evocative morphology. The colony diameter may be from 0.5 mm to 2 mm after 4 days of culture in an adequate atmosphere;
- the strain is a small Gram-negative coccobacillus;
- it agglutinates an anti-Brucella specific serum
- it is oxidase and/or urease positive

11 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 the specimen 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 temperature ≤ –16°C in tryptose broth with 15% (v/v) glycerol. For the maintenance of strains as well as for sending them to a reference laboratory for typing, it is essential that only smooth colonies be selected.

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

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 (6.3.1) 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.

12 Restitution of results

For laboratory's clients, restitution of the results is made, for example, as follows:

Diagnosis of *Brucella* by bacteriological culture:

- "Absence of *Brucella* after 10 days of culture" or "No isolation of *Brucella* after 10 days of culture";

or,

- "Isolation of a suspected *Brucella* strain" or "Isolation of a strain presenting the specific characteristics of a *Brucella*" or "Isolation of a strain the characteristics of which cannot exclude *Brucella*";

and,

- "The strain was transferred to the reference laboratory for confirmation";

or,

"Presence of numerous contaminants that could hinder the isolation of *Brucella*. Inconclusive result".

13 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 tests conditions. The expected property of this reference or control strain must be effectively found.

14 Analysis report

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