IMMUNOLOGICAL DIAGNOSIS OF BRUCELLA ABORTUS
IN BOVINE MILK SAMPLES

BY ELISA METHOD

960 REACTIONS

POURQUIER®ELISA Bovine Brucellosis Milk Screening
VERSION : P04210/09 - 05-10/2006
INTRODUCTION

Brucellosis is a zoonosis found throughout the world with major implications both in the field of public health and farming economy. It is most often caused by Brucella abortus (cattle) or Brucella melitensis (small ruminants). It causes abortions, and the bacteria are excreted in the milk. Humans are contaminated by contact with or ingestion of infected products (milk, cheese, meat). The main signs of infection are undulant fever, with serious and frequent complications during a chronic evolution. The extent of this disease has led health authorities to implement screening programs to detect infected animals and to eradicate the disease. Brucellosis screening is mainly done with serological tests (Rose Bengal Test, Wright’s Serum Agglutination Test, Complement Fixation Test, Ring-Test and ELISA) which are the only tests applicable to mass screening programs. Among the serological techniques used, the ELISA method is the most recent application. This technique allows the analysis of milk samples, and it is particularly well suited to automation and it shows a very good sensitivity and often a better specificity than the Ring-Test.

PRINCIPLE OF THE TEST

The principle of the test is:

1) All the wells of the polystyrene microplates are coated with the LPS of Brucella abortus.
2) Milks samples are diluted and incubated in the wells. Any antibodies specific to Brucella present in the milk will form a LPS-antibody immune-complex and remains bound in the wells.
3) After washing, a Peroxidase conjugated anti-ruminant IgG is added to the wells. This conjugate will bind to the immune-complex.
4) After another washing, the enzyme substrate (TMB) is added to the conjugate, forming a blue compound becoming yellow after blocking. The intensity of the colour is a function of the antibody concentration in the milk sample to be tested.

The limit of positivity is set with a control milk, which must be added to each microplate. This reagent is standardised with the OIEISS serum and designed to give results according to the requirements of the European Directive (CEE 64/432 modified on December 11th 1984, June 26th 1991 and March 2002).

PRECAUTIONS FOR USE

1) Do not place the pipette in the mouth when testing reagents.
2) Avoid contact of the substrate (TMB*) with skin, mucous membranes and eyes.
3) “Stop-solution” contains $\text{H}_2\text{SO}_4$*(0,5M) acid, that can cause serious burns in case of contact with skin, mucous membranes and eyes.
4) Even if the material in the package does not contain any contaminating element, and that the sera are, in theory, non-infectious, it is nevertheless advised to decontaminate the whole disposable elements, either by immersion for at least 1 hour in freshly prepared 5% sodium hypochlorite, or by autoclaving them at 120°C for a minimum of 1 hour or by any other method in accordance with the reglementation in force, before discarding.

* The toxicity form of the products is available at the Institut Pourquier.
MATERIALS REQUIRED BUT NOT INCLUDED IN THE KIT

1) Microplates reader  
2) Centrifuge  
3) Centrifuge tubes and microtubes  
4) Vortex or similar  
5) Microplate washing system that distributes 300 µl per well  
6) Precision Micropipettes and Multi-dispensing micropipettes (The precision required must be lower or equivalent to 5% of the volumes indicated)  
7) Disposable pipette tips  
8) Distilled water : the water used for the reconstitution of controls and of the wash solution can be produced by a conventional distillation system or any other high-performance water purification system (reverse osmosis, resin or activated charcoal purification …).  
9) Microplate covers (lid, aluminium foil or adhesive)

KIT CONTENTS and STORAGE OF REAGENTS

It is recommended to bring at room temperature 21°C (± 5°C) all the reagents of the kit at least one hour before use (except the conjugate)

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<th>COMPONENTS</th>
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| Monowell Coated microplates | 10 | +5°C (± 3°C)  
• If a microplate is not entirely used, it may be stored for later use during 3 months if it is immediately closed in an airtight container and stored at +5°C (± 3°C). |
| Concentrated (X 20) wash solution | 2 x 100 ml bottles | +5°C (± 3°C)  
• May form crystals at +5°C (± 3°C), which rapidly disappear at +21°C (± 5°C). A gentle shaking of the solution will accelerate the dissolution of the crystals  
• This solution can also be stored at +21°C (± 5°C) for 1 month, if the vials are closed in an airtight way, in order to be immediately available when needed.  
• The "Concentrated (X 20) wash solution" is the same for all the kits of the INSTITUT POURQUIER and can be used equally in the different kits.  
• After dilution, the Wash Solution can be stored for 3 days at +5°C (± 3°C) |
| Dilution Buffer 1 Sky blue | 3 x 120 ml bottles | +5°C (± 3°C) |
| Positive control (freeze-dried) | 2 x 1 ml bottles | +5°C (± 3°C)  
• After reconstitution, the positive and the negative control milks must be stored in aliquots at a temperature ≤ −16°C  
• They can be frozen and unfrozen up to three times with no loss of activity. A storage at +5°C (± 3°C) will lead to a significant increase of the background noises |
| Negative control (freeze-dried) | 2 x 1 ml bottles | +5°C (± 3°C)  
• After reconstitution, the positive and the negative control milks must be stored in aliquots at a temperature ≤ −16°C  
• They can be frozen and unfrozen up to three times with no loss of activity. A storage at +5°C (± 3°C) will lead to a significant increase of the background noises |
| Monoclonal anti-ruminant IgG / Peroxidase conjugate | 1 x 1,5 ml bottle | +5°C (±3°C)  
• The diluted conjugate can be stored 2 hours at +21°C (±5°C) |
| Revelation solution 3 (TMB) Ready to use | 1 x 120 ml bottle | +5°C (±3°C)  
• This solution can be slightly bluish at +5°C (±3°C) and becomes colourless at +21°C (±5°C).  
• Thus it can be left on the draining board at +21°C (±5°C) up to 1 week (if the vial is well closed in an airtight way), in order to be immediately available when needed. |
| Stop Solution (H₂SO₄ 0,5M solution) | 1 x 120 ml bottle | +5°C (±3°C)  
• It can be stored at +21°C (± 5°C ) up to 1 month if the vial is well closed in an airtight way), in order to be immediately available when needed.  
• The "Stop Solution" is the same for all the kits of the INSTITUT POURQUIER and can be used equally in the different kits. |

Using Instructions
INSTRUCTIONS FOR USE

1) DEPOSITING THE MILK SAMPLES

a - Reconstitution of the controls:
- Reconstitute the positive control milk with 1 ml of distilled water.
- Reconstitute the negative control milk with 1 ml of distilled water.

b - Milk samples:
- The samples (controls and samples to be analysed) are diluted at 1/5 in "Dilution Buffer 1".
- Dispense:
  - 200 µl of "Dilution Buffer 1" per well
  - 50 µl of undiluted negative control milk in A1
  - 50 µl of undiluted positive control milk in B1 and C1
  - 50 µl per well of each milk sample to be tested (following figure 1: only one well per sample to be tested).
- Homogenize the contents of the wells by gently shaking the plate.
- Cover the plate (with a lid, aluminium foil or adhesive)
- Incubate for **1 hour and 30 min. (+5 min) at +21°C (+5°C)**

Note:
1) It is also possible to prepare a 1/5 dilution of the controls and of the samples in the "Dilution Buffer 1", and then to dispense:
   - 250 µl of 1/5 diluted negative control milk in A1
   - 250 µl of 1/5 diluted positive control milk in B1 and C1 (see notes 1 & 2)
   - 250 µl per well of each 1/5 diluted milk sample to be tested (only one well per sample to be tested).

2) Milk samples may be skimmed or full fat milk.

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Figure 1: Deposit of the samples

Notes:
1) The individual filling of the 96 wells is sometimes a long process. In order to standardize the milk incubation time, the controls and milk samples are prepared in 96-well plates with U-shaped bottoms. It is therefore possible to transfer them rapidly (row by row) by using a multi-channel pipette. The dilutions of samples must be done in the same way as the dilution of control.
2) The position of control milks in A1 and B1 and C1 is not important. They may be dispensed anywhere on the plate.
3) Laboratories using automatic methods may not have enough reagents (i.e., "concentrated X20 wash solution" or "Dilution Buffer"). Extra reagents can be supplied free of charge, on request.
4) Milk controls and samples diluted in "Dilution Buffer 1" can be stored up to 3 hours at +21°C (+5°C)
2) **WASHING**

a) Dilute a vial of "Concentrated (X 20) Wash Solution" in 1900 ml of distilled water. This solution is hereafter called "Wash solution". The dilution can be carried out before the elimination of the crystals appeared at +5°C (±3°C), if the whole 100 ml vial is used.

b) Empty the content of the plate by a «flick-off» method or better by a manual or automatic method.

c) Fill all the wells on the plate with the "wash solution", then empty them again.

d) Repeat step "c" twice (a total of 3 washes).

**Notes:**

1) If milks are skimmed (or taken under the cream), this type of washing is sufficient. With full fat milk, it can be necessary to modify this method of washing: if a visual control reveals whitish traces in the wells, it is recommended to leave the wash solution in contact 1 to 3 times 3 minutes. Indeed, these times of contact allow the elimination of fat particles that are likely to fix in a non-specific way the conjugate in the next step.

2) When several plates are processed at the same time, it is possible (in order to synchronize all the steps) to leave the plates full of "wash solution" during one hour without modifying the validity of the test.

3) **DEPOSITING THE CONJUGATE**

a) Dilute the conjugate to 1/100 in "Dilution Buffer 1"

b) Dispense 100 µl per well of this diluted conjugate

c) Cover the plate (with a lid or aluminium foil) and incubate for **30 minutes** (± 3mn) at +21°C (±5°C)

4) **WASHING**

a) Empty the content of the plate by a «flick-off» method or better by a manual or automatic method.

b) Fill all the wells with the wash solution, then empty them again.

c) Repeat step (b) twice (a total of 3 washes).

**Notes:**

1) Particular care with the last wash is very important in getting a good test result.

2) If the washing is carried out with a manual method, it is possible after the last washing to drum the microplate on an absorbent support in order to empty the wells completely.

5) **REVELATION**

a) Dispense 100 µl of "Revelation Solution 3" ready to use per well

b) Incubate the plate at +21° (± 5°C). for 20 minutes (away from the light).

c) Dispense 100 µl of "Stop Solution" per well.

d) Shake gently the plate until the coloured solution is homogenized. Wipe carefully the underside of the plate.

**Notes:**

1) The 20 – minute revelation period, which is indicated in the method, gives the O.D. values provided in the paragraph "INTERPRETATION", when implemented in our laboratories. However the rate of colour revelation can be slightly affected by different factors (quality of the washes, quality of water used, precision of the pipetting, temperature of the reaction...). Regarding the work conditions, the revelation step may give OD values higher or lower than those expected. So, the user may stop the reaction after 20 minutes (±10 min).

2) The reading can be done up to 1 hour after having stopped the reaction on condition that the plates are kept in the dark.

6) **READING**

Read the optical densities at 450 nm (OD.450). The photometer must first be blanked on air.
VALIDATION CRITERIA

The reaction is considered valid when the following criteria are obtained:

- The positive control milk has a minimal mean OD 450 value of 0.400

and

- If a ratio between the OD 450 value of the positive control milk and OD 450 value of the negative control milk is greater than or equal to 3.

INTERPRETATION

Calculate for each sample, the ratio S/P (%):

\[
S/P\% = 100 \times \frac{\text{OD450 value of the sample} - \text{OD450 value of the negative control}}{\text{mean OD450 value of the positive control} - \text{OD450 value of the negative control}}
\]

If the validation values are obtained, the status of the tested milks is as follows:

- Sample with a S/P % lower than or equal to 45% are considered coming from a herd, which has not been in contact with Brucella abortus.
- Sample with a S/P % between 45% and 55% are considered to be doubtful. A second test is necessary to determine the status of these milks.
- Sample with a S/P % greater than or equal to 55% are considered coming from a herd, in which at least one animal has been in contact with Brucella abortus.

BIBLIOGRAPHY


LEGEND

 Modification in the using instructions