SEROLOGICAL DIAGNOSIS OF SHEEP AND GOAT BRUCELLOSIS

BY ELISA METHOD

ANALYSIS OF INDIVIDUAL SERUM AND PLASMA SAMPLES

(960 Reactions)

POURQUIER® Elisa Sheep and Goat Brucellosis Screening

VERSION: P04310/07
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 (bovines) or Brucella melitensis (small ruminants). It causes abortions and the bacteria are excreted in the milk. Man is contaminated by contact with or ingestion of infected products (milk, cheese, meat). The main signs of contamination 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 shows a very good detectability, it is applicable to individual sera and plasma. This one is particularly well suited to automation.

PRINCIPLE OF THE TEST

The principle of the test is:

1) The wells of the microplates are coated with Brucella LPS (see note 1).

2) Samples are diluted and incubated in the wells. Any antibodies specific to Brucella present in the samples will form a LPS-antibody immune-complex and remain 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 measure of the rate of antibodies present in the samples to test.

The cut-off is set by using a positive control (see note 2), which must be added to each microplate.

Notes:

1) When used in ELISA methods for the analysis of polyclonal samples of ruminants, the LPS of Brucella melitensis and the LPS of Brucella abortus do not present visible difference between them (see references 3 and 4)

2) There is no international standard for the determination of detectability for ELISA for small ruminants. The cut-off proposed in this kit has been defined by the analysis of a population of positive and negative samples coming from well-known herds. Analysed in agglutination, this cut-off corresponds to a serum situated at around 15 International Units.

PRECAUTIONS FOR USE

1. Do not place the pipette in the mouth when testing reagents.

2. “Stop-solution” contains H₂SO₄*(0,5M) acid, that can cause serious burns in case of contact with skin, mucous membranes and eyes.

3. Even if the material in the package does not contain any contaminating element, and that the samples 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 product is available at the Institut Pourquier
**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).

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY</th>
<th>STORAGE AND NOTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monowell Coated microplates</td>
<td>10</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 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).</td>
</tr>
<tr>
<td>Concentrated wash solution (X 20)</td>
<td>2 x 100 ml bottles</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 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</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• This solution can also be stored at +21°C (± 5°C) for 1 month, if the vials are closed in an airtight way, and be immediately available when needed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Identical for all the kits of the INSTITUT POURQUIER and can be used equally in the different kits.</td>
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<tr>
<td></td>
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<td>• After dilution, the Wash Solution can be stored for 3 days at +5°C (± 3°C)</td>
</tr>
<tr>
<td>Dilution Buffer 2 Light green</td>
<td>2 x 120 ml bottles</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td>Dilution Buffer 1 Light blue</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td>Positive control</td>
<td>1 x 1 ml bottle</td>
<td>-5°C (± 3°C)</td>
</tr>
<tr>
<td>Negative control</td>
<td>1 x 1 ml bottle</td>
<td>-5°C (± 3°C)</td>
</tr>
<tr>
<td>Monoclonal anti-ruminant IgG / Peroxidase conjugate</td>
<td>1 x 1.5 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The diluted conjugate cannot be stored</td>
</tr>
<tr>
<td>Revelation solution 2 (TMB) Ready to use</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td>Stop Solution (H₂SO₄ 0.5M solution)</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• It can be stored at +21°C (± 5°C) up to 1 month (if the vial is well closed in an airtight way) and be immediately available when needed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Identical for all the kits of the INSTITUT POURQUIER, it can be used equally in the different kits.</td>
</tr>
</tbody>
</table>

Using Instructions
MATERIALS REQUIRED BUT NOT INCLUDED IN THE KIT

1) Microplate spectrophotometer
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% for all the volumes indicated)
7) Disposable pipette tips
8) Distilled water: The water used for the dilution of the concentrated wash solution may 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)

INSTRUCTIONS FOR USE

1) DISTRIBUTION OF THE SAMPLES

Samples are diluted to 1/20 using the following method:
- Dispense:
  - 190 µl of "dilution buffer 2" per well.
  - 10 µl of undiluted negative control serum in A1
  - 10 µl of undiluted positive control serum in B1 and C1
  - 10 µl per well of each undiluted sample to test (only one well per sample to test):
- Homogenize the contents of the wells by gently shaking the plate (see note 1).
- Cover the plate (with a lid, aluminium foil or adhesive).
- Incubate for **45 min. (±5 min) at +21°C (± 5°C)**.

![Figure 1: Distribution of the samples](image)

Notes:
1. In our laboratories, a microplate shaker originally designed for a complement fixation test micromethod, is used.
2. The individual filling of the 96 wells is sometimes a long process. In order to standardize the sample incubation time, the controls and samples are prepared in plates with 96 U-shaped-bottom-wells. It is therefore possible to transfer them rapidly (column by column) using a multi-channel pipette. **The dilutions of samples must be done in the same way as the dilution of controls.**
3. The position of controls in A1, B1 and C1 is not important, they may be dispensed anywhere on the plate.
2) WASHING

a) Dilute a vial of “concentrated wash solution (X 20)” in 1900 ml of distilled water. This solution is hereafter called “wash solution”. The dilution can be carried out before the disappearance of the crystals appeared at + 5°C (± 3°C) on condition that the whole 100 ml vial is used.
b) Empty the content of the plate by “flick-off” or better by another manual or automatic method.
c) Fill all the wells of the plate with the wash solution; then empty them again.
d) Repeat step c) twice (a total of 3 washes).

Note: 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 » up to one hour with no result on the validity of the test.

3) DEPOSITING THE CONJUGATE

a) Dilute this conjugate to 1/100 with the "Dilution buffer 1".
b) Dispense 100 μl of the diluted conjugate in each well.
c) Cover the plate (with a lid, aluminium foil or adhesive) and incubate for 30 minutes (±3 min.) at +21°C (±5°C).

4) WASHING

a) Empty the content of the plate by “flick-off” or by some other manual or automatic method.
b) Fill all the wells on the plate with the wash solution; then empty them again.
c) Repeat the step b) twice (total of 3 washes)

Note:
1. Particular care with the last wash is very important in getting a good test result.
2. If the wash is carried out manually, the microplate may be tapped gently on an absorbent support after the last wash in order to empty the wells completely.

5) REVELATION

a) Dispense 100 μl of “Revelation Solution Z’ ready to use per well
b) Incubate the plate at +21°C (± 5°C). For 10 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

Note:
1. The 10-minute-revelation period as indicated in the method, when performed in our laboratories give the O.D. values provided in the paragraph "Validation Criteria”. 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...). So, the user may stop the reaction at 20 minutes ± 5 minutes.
2. The reading can be done up to 1 hour after blocking if the microplates are stored away from the light.

6) READING

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

The results can be considered reliable if:

- The positive control serum has a minimal **OD.450 value of 0.350** and
- The ratio between the **OD.450 value of the positive control and OD.450 value of the negative control** is greater than or equal to 3.

INTERPRETATION

Calculate for each sample the S/P percentage:

\[
S/P\% = 100 \times \frac{\text{OD.450 of the sample} - \text{OD.450 of the negative control}}{\text{OD.450 of the mean positive control} - \text{OD.450 of the negative control}}
\]

- Samples whose S/P% is **equivalent or lower than 110%** are considered to be from an animal which does not have specific antibodies of the Brucella LPS.
- Samples whose S/P% is between **110% and 120%** are considered to be doubtful. A second determination is necessary to determine the status of these samples.
- Samples whose S/P% is **equivalent or greater than 120%** are considered to be from an animal which has specific antibodies of the Brucella LPS.

**Note:**
1. Since ELISA has a higher sensitivity than other conventional methods, the positive reaction will not always be confirmed by Wright, Rose Bengale or FC’ tests.
2. If adult animals were vaccinated less than one month ago, it is possible to observe antibody traces indicated by an increased background or transiently weakly positive results on some animals.

BIBLIOGRAPHY


LEGEND

**ζ** = Modification in the using instructions