SEROLOGICAL DETECTION OF SPECIFIC ANTIBODIES TO Mycoplasma mycoides subspecies mycoides Small Colony (MmmSC), THE ETIOLOGIC AGENT OF CONTAGIOUS BOVINE PLEUROPNEUMONIA (CBPP)

(800 REACTIONS)

CBPP serum competition ELISA
Version: P05410/02
INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is an infectious disease listed by the OIE. It is due to a mycoplasma: *Mycoplasma mycoides* subsp. *mycoides* small colony biotype (MmmSC). In its acute form it is manifested by anorexia and respiratory symptoms (coughing, nasal discharge, dyspnoea, polypnoea) and by the presence of exudative pleurisy and pneumonia. Some animals develop sub-acute forms with very limited and hardly detectable symptoms. Post-mortem examination often reveals sequestra: encapsulated pulmonary lesions in which the content necroses progressively. Since these animals may act as silent carriers, they represent a major transmission risk. The transmission of CBPP occurs by direct contact between infected and healthy animals.

The control of the disease in countries where the prevalence is low is based on the detection of infected herds and the systematic elimination of all the animals in an infected herd. Every movement of animals around the infection source is controlled. In other countries, mainly in Africa, vaccination programs with attenuated vaccines, such as T1/44 and T1-sr, are used for CBPP control.

CBPP infections may be clinically suspected upon appearance of respiratory symptoms, although these are not pathognomonic. The suspicion may also come either from the observation of evocative lesions at necropsy or from routine controls at slaughter. In any case, confirmation from a diagnostic laboratory is needed. This may be carried out by culture and identification of MmmSC from suspicious lesions, although this may be difficult to obtain, particularly when animals are treated with antibiotics. The confirmation may also be obtained by serological tests in the suspicious herd.

The OIE reference method for CBPP serology is the complement fixation test (CFT). This technique has been used for CBPP eradication campaigns in many countries. However, it presents some disadvantages, particularly due to the existence of non-specific positive results and to difficulties encountered in the standardization of antigen production.

For these reasons CIRAD-UMR15 (FAO and OIE world reference centre for CBPP) has developed another test, a competition ELISA (c-ELISA) based on a monoclonal anti-MmmSC antibody, named Mab 117/5. This test is an alternative to the CFT for the OIE and can be used for official CBPP testing.

It must be noted that, for both c-ELISA and CFT, the results must be interpreted at the herd level. The animals in the incubation stage cannot be detected. This is also true for many animals in the chronic stage of the disease, since the percentage of positive animals decreases progressively after infection. Consequently, the sampling protocol should be adapted to this situation and a significant number of animals must be tested in the suspicious herd. It is advisable to give priority to animals showing respiratory symptoms a few weeks prior to sampling.

Vaccination with strains such as T1/44 or T1-sr does not always induce detectable antibody responses. Therefore, it is not possible to use CFT or c-ELISA to assess vaccination efficiency. However, as post-vaccinal antibodies do not persist after 3 months, CFT or c-ELISA can be used for the detection of natural infections, even in areas where vaccination is used.

This kit has been evaluated by the Joint Division FAO/AIEA within the framework of a Coordinated Research Project (CRP).

PRINCIPLE

The principle of the test is:

1) The microplates wells are coated with an MmmSC lysate.

2) Serum samples to be tested are diluted and mixed with the specific monoclonal antibody (Mab 117/5) in a dilution plate or “pre-plate”. This mixture is then transferred into the MmmSC-coated microplate. Any specific antibodies present in the test sera will bind to the MmmSC antigen, competing with the Mab for the specific epitope.

3) After washing, an anti-mouse IgG serum conjugated to horseradish peroxidase (HRP), which will bind to any Mab fixed to the wells, is added. If specific MmmSC antibodies are present in the bovine sera, they will displace the Mab and the conjugate will not be able to bind.

4) Following another series of washes, the HRP substrate (TMB) is added, forming a blue compound that will turn to yellow when the reaction is stopped. The intensity of the color is an inverse measure of the proportion of MmmSC antibodies present in the test sera.
The cut-off point is calculated using the results obtained from a monoclonal control (Cm, 0% inhibition) and a conjugate control (Cc, 100% inhibition). Positive and negative control sera are delivered within the kit. They must be included in each microplate in order to validate the results.

**DETECTABILITY**

Analyses of sera obtained from CBPP-free areas showed that the percentage of inhibition obtained from those areas were generally near 25% and never exceeded 50%. Therefore, the cut-off point was fixed at 50% and was validated for sera from different geographic origins. The specificity of the test was estimated at 99.9%.

In experimentally infected herds (acute stage) all the animals that had shown lesions were seropositive at least once. Some of the animals that did not show any lesions also gave positive results and were identified as MmmSC carriers. The persistence of detectable antibodies varied from one animal to another. The detectability of antibodies was optimal during the first month after infection. The percentage of positive animals then decreased during the following 6 months.

**Beware:** The test sera must not have been treated by heat (for example for decomplementation). Otherwise, they may provide false negative results by c-ELISA.

**Note:** Since this test is a blocking ELISA it can theoretically be used to test sera coming from any species. It has been tested experimentally using sera coming from goats, which can, in certain circumstances, be MmmSC carriers. The results obtained were similar to those observed for cattle. Nevertheless, if this test was to be used for sera of species other than cattle, it should be previously validated by a trial including a significant number of animals coming from a CBPP-free area.

**MATERIALS REQUIRED BUT NOT PROVIDED**

1) Refrigerator +5°C (± 3°C) and freezer ≤ -16°C
2) Incubator at +37°C (±3°C)
3) Plate agitator
4) Microplate reader
5) Microplate washing system that distributes 300 μl per well (optional)
6) Vortex or similar (optional)
7) Precision micropipettes and multichannel micropipettes (the precision required must be lower than or equivalent to 10% for volumes lower or equal to 10 μl and to 5% for all the other volumes indicated)
8) Disposable micropipette tips
9) Reagent reservoirs for multichannel pipettes
10) 96-well microplates for dilutions
11) Microplate covers (lid, aluminium foil or adhesive)
12) Distilled water. The water used for the reconstitution of controls and of 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...)
13) Disinfectant for safe disposal of test sera (when required)

**SAFETY MEASURES**

1) Do not perform mouth-pipetting.
2) “Stop solution” contains 0.5M H₂SO₄*, an acid that can cause serious burns in case of contact with skin, mucous membranes or eyes.
3) Avoid contact of the substrate (TMB*) with skin, mucous membranes and eyes.
4) Control sera contain sodium azide* and may be toxic if ingested.

*Institute POURQUIER is ready to supply the safety data sheet of this product on demand.
KIT CONTENTS AND STORAGE

It is recommended to work with reagents at +21°C (± 5°C). Thus, it is advisable to bring all kit components (except the conjugate) to room temperature at least one hour before the beginning of the test.

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY</th>
<th>STORAGE AND NOTES</th>
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<tr>
<td>96-well coated microplate</td>
<td>10</td>
<td>+5°C (±3°C)</td>
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<tr>
<td>Wash concentrate (20x)</td>
<td>2 x 100 ml bottles</td>
<td>+5°C (±3°C) • May form crystals at +5°C (± 3°C), although these rapidly disappear at +21°C (± 5°C). Gentle shaking will accelerate the dissolution of the crystals. This solution can also be stored at +21°C (±5°C) for up to 1 month, as long as the containers are closed airtight, so as to be immediately available when needed. • After dilution the “wash solution” can be stored for 3 days at +5°C (±3°C). • The “Wash Concentrate (20x)” is the same for all the kits produced by the INSTITUT POURQUIER and can be used indistinctively for the different kits.</td>
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<td>Dilution buffer</td>
<td>3 x 120 ml bottles</td>
<td>+5°C (±3°C)</td>
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<td>Strong positive control (CP++)</td>
<td>1 x 0.5 ml vial</td>
<td>+5°C (±3°C) • If the plates are not used within a week from opening, the positive and negative control sera must be stored in 100μl- aliquots at a temperature ≤ -16°C.</td>
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<td>Weak positive control (CP+)</td>
<td>1 x 0.5 ml vial</td>
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<td>Negative control (CN)</td>
<td>1 x 0.5 ml vial</td>
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<tr>
<td>Monoclonal anti-MmmSC antibody (Mab 117/5) (freeze-dried)</td>
<td>1 x 1 ml vial</td>
<td>• After reconstitution, the monoclonal antibody must be stored in aliquots at a temperature ≤ -16°C.</td>
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<tr>
<td>Anti-mouse IgG HRP-conjugated</td>
<td>1 x 1.2 ml vial</td>
<td>+5°C (±3°C) • The diluted conjugate solution cannot be stored.</td>
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<td>TMB substrate Solution 3</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (±3°C)</td>
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<tr>
<td>Stop solution (0.5M H₂SO₄ solution)</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (±3°C) • It can also be stored at +21°C (± 5°C) for up to 1 month (if the bottle is closed airtight) in order to be immediately available when needed. • Identical for all the kits of the INSTITUT POURQUIER, it can be used indistinctively in the different kits.</td>
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INSTRUCTIONS FOR USE

1) DISTRIBUTION OF TEST AND CONTROL SERA

Prior to transferring the sera into the coated microplate, they are diluted and mixed with the monoclonal anti-MmmSC antibody (Mab 117/5) in a 96-well microplate , called the “pre-plate”, which should be made out of normal plastic without any absorption activity (fig. 1).
**Figure 1: Distribution of sera and Mab**

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Cc: Conjugate control (without serum, without Mab = 100% inhibition)
Cm: Monoclonal control (without serum = 0% inhibition)
CP+: Strong positive serum
CP++: Weak positive serum
CN: Negative serum
1: Test serum # 1
2: Test serum # 2
3: ...

**a) Distribution of controls and test sera**

These dilutions must be carried out just before the test:
- Distribute 100 μl “Dilution buffer 24” in all the wells of the pre-plate.
- Distribute 110 μl “Dilution buffer 24” in wells A1 and A2 (Conjugate control, Cc)
- Distribute 11 μl of the three control samples: CP++ in B1, B2, C1, C2 and CP+ in D1, D2, E1, E2 and CN in H1, H2.
- Distribute 11 μl of each of the test sera in the remaining wells (A3 to H12, as required).

**b) Reconstitution and distribution of the monoclonal antibody (Mab 117/5)**

Reconstitute the Mab 117/5 with 1 ml of distilled water. Dilute the necessary quantity of the Mab to 1/120 in “Dilution buffer 24” (i.e.: 100μl Mab in 11.9 ml “Dilution buffer 24” for one plate).
- Distribute 110 μl diluted Mab 117/5 in all the wells except for A1 and A2.

**Note:** If the whole amount is not immediately used, the monoclonal antibody must be stored in aliquots at a temperature ≤-16°C (before dilution in “Dilution buffer 24”).

**c) Incubation of the serum/Mab mixture**

- Mix by pipetting and transfer 100 μl of the serum/Mab mixture from the pre-plate into the coated plate by using a multi-channel pipette (see fig. 1).
- Cover the plate with a lid, aluminium foil or adhesive and incubate for 1 hour (+/- 5 minutes) at 37°C (±3°C) under gentle agitation.

**2) WASHING**

**a) Dilute a bottle of “Wash concentrate (20x)” in 1900 ml of distilled water. This solution is hereafter called the “wash solution”. The dilution can be carried out before the disappearance of the crystals which may appear at +5°C (±3°C) as long as the whole 100 ml bottle is used. However, if the whole amount is not to be used immediately, prepare only 400 ml of wash solution per plate tested (i.e.: dilute 20 ml “Wash concentrate (20x)” in 380 ml water).

**b) Empty the contents of the plate by inversion or by another manual or automatic method.

c) Fill all the wells of the plate with wash solution; then empty them again.

d) Repeat step c) (a total of 2 washes).

**Note:** When performing the washes manually, empty the plates completely by tapping them upside-down on an absorbent towel.

**3) DISTRIBUTION OF THE CONJUGATE**

**a) Dilute the conjugate to 1/100 in “Dilution buffer 24” (i.e.: 120 μl conjugate in 11.88 ml “Dilution buffer 24” for one plate).

**b) Distribute 100 μl of diluted conjugate in each well.**
c) Cover the plate with a lid, aluminium foil or adhesive and leave to incubate for **30 minutes (± 3 minutes)** at **37°C (±3°C)** under gentle agitation.

4) **WASHING**  
   a) Empty the content of the plate by returning or by another manual or automatic method.  
   b) Fill all the wells of the plate with the wash solution; then empty them again.  
   c) Repeat step b) twice (a total of 3 washes).  

   **Note:** The care brought to the last washing is essential for a good implementation of the test. If the washing is manual, the plate can be inverted and tapped on an absorbent support, in order to completely empty the wells after the last washing.

5) **REVELATION**  
   a) Distribute 100 µl of ready to use"TMB substrate 3" per well.  
   b) Cover the plate and incubate for **30 minutes (± 10 minutes) at +37°C (±3°C)** under gentle agitation.  
   c) Add 100 µl of "Stop solution" per well.  
   d) Gently shake the plate until the colored solution is homogenized. Wipe carefully the bottom of the plate.  

   **Notes:**  
   1. A 30-minute incubation with the substrate provides the O.D. values given in the paragraph "INTERPRETATION" when implemented in our laboratories. However, the rate of color development can be slightly modified by different factors (quality of the washes and of the water used, pipetting precision, temperature of the reaction...). Depending on working conditions, assay development may result in OD values higher or lower than those expected. Therefore, the user may stop the reaction after 30 minutes ± 10 minutes.  
   2. Reading may be performed up to 1 hour after the reaction has been stopped as long as the plates are kept in the dark.

6) **READING**  
   a) Read the optical densities at 450 nm (OD450). The photometer could first be blanked on air.  
   b) Calculate the mean value of the **Cm** (0% inhibition) and **Cc** (100% inhibition) controls.  
   c) Calculate the percentage of inhibition (PI) for each serum as follows:  

   \[ PI = 100 \times \frac{(OD \text{ Cm} – OD \text{ Test})}{(OD \text{ Cm} – OD \text{ Cc})} \]

   **VALIDATION CRITERIA**  
   The reaction is considered valid when the following criteria are obtained:  
   → The OD of **Cm** must be between 0.5 and 2.0 (preferably near 1.0)  
   → The OD of **Cc** must be below 0.3  
   → The PI of CN must be equal to or lower than 35%  
   → The PI of CP+ must be between 50 and 80%  
   → The PI of CP++ must be between 60 and 90%  

   **INTERPRETATION**  
   • Sera with a percentage of inhibition equal to or lower than **40%** are considered negative.  
   • Sera with a percentage of inhibition between **40 and 50%** are considered doubtful.  
   • Sera with a percentage of inhibition equal to or greater than **50%** are considered positive.  

   **LITERATURE**  
**LEGEND**  

Edition: Modification in the operating procedure.