SEROLOGICAL DIAGNOSIS OF SPECIFIC ANTIBODIES TO THE VP7 PROTEIN OF THE BLUETONGUE VIRUS BY COMPETITIVE ELISA METHOD IN SERUM AND PLASMA

(480 Reactions)
INTRODUCTION

Bluetongue is an infectious non-contiguous viral disease of sheep, goats, cattle and wild ruminants. It is not transmissible to man and is an OIE list A disease. The outcome of the infection ranges from inapparent (cattle and goat) to fatal with high mortality rates (sheep). The disease is caused by the Bluetongue Virus (BTV), which is a member of the Orbivirus genus of the family Retroviridae, and transmitted by a hematophagic fly of the Culicoides genus. Direct contagion from one animal to another is not possible. The geographic extension is limited to areas where the vector can survive, that means a large zone between the 40th degree north to 35th degree south, which is expanding at present (Corsica and Sardinia are concerned since 2001).

The clinical signs of the disease, when they appear, include a febrile response characterised by inflammation and congestion, facial oedema and haemorrhages and ulceration of the mucous membranes. In severe cases the tongue may show intense hyperaemia (where the name of the disease comes from), become swollen and oedematous and protrude from the mouth. There is often severe muscle degeneration and mortality up to 15%.

A similar severe disease of wild ruminants is caused by the epizootic haemorrhagic disease virus (EHDV), which, like the BTV is a member of the Orbivirus genus, but is classified in a separate serogroup. The EHDV occasionally cause clinical signs in cattle that appear to be similar to bluetongue. Within the Orbivirus there are 14 different serogroups and the bluetongue serogroup contains 24 serotypes. Most of the serogroups appear to be immunologically distinct, but there is considerable cross-reaction between members of the BT and EHD serogroups.

Serological responses in ruminants appear some 7-14 days after BTV infection and are generally long lasting.

Until recently, tests such as agar gel immunodiffusion and indirect ELISAs were used to detect BTV serogroups specific antibodies although these tests had the major drawback of being unable to consistently distinguish between antibodies to viruses in the BT and EHD serogroups. Competitive ELISAs in the contrary can resolve this problem and are now recommended.

The proposed test can detect BTV specific antibodies in individual sheep, goat and cattle sera and plasma.

It is based on the competition between the samples to be tested and a monoclonal antibody, which is coupled to the peroxidase. This monoclonal antibody is directed to the N-terminal part of the VP7 protein, a major core protein of the BTV (specific for the BT serogroup).

This method is easy to implement, rapid and reliable and is particularly suited to the analysis of a large number of samples.

PRINCIPLE OF THE TEST

The principle of the test is:

1) All the wells of the microplate are coated with recombinant VP7 protein.
2) Samples to be tested are diluted and incubated in the wells. Any antibody specific to VP7 present in the samples will form an antigen-antibody immune-complex.
3) After incubation, an anti-VP7 antibody coupled to the peroxidase is added in the wells. If the sample contains specific VP7 antibodies, the VP7 sites are “masked” and the conjugate cannot bind on the corresponding epitope. On the contrary, the conjugate can bind on the VP7.
4) After washing, the enzyme substrate (TMB) is added to the conjugate, forming a blue compound becoming yellow after blocking. The intensity of the colour is an inverse measure of the proportion of anti-VP7 antibodies present in the sample to test.

The cut-off is set by using a negative control, which does not induce any extinction and has to be added to each microplate.
**KIT CONTENTS AND STORAGE**

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 and the controls).

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY</th>
<th>STORAGE AND NOTES</th>
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<tbody>
<tr>
<td>Monowell Coated microplates</td>
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<td>+5°C (± 3°C)</td>
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<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>
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<tr>
<td>Concentrated (20x) wash solution</td>
<td>1 x 100 ml bottle</td>
<td>+5°C (± 3°C)</td>
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<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. This solution can also be stored at +21°C (± 5°C) up to 1 month, if the vials are closed in an airtight way, in order to be immediately ready to use when needed.</td>
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<td>• The &quot;Concentrated (X 20) wash solution&quot; is the same for all the kits of the INSTITUT POURQUIER and can be used equally in the different kits.</td>
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<td>• After dilution, the wash solution can be stored for 3 days at +5°C (± 3°C)</td>
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<td>Dilution Buffer 2 Light green (for samples)</td>
<td>1x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
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<td>• Shake before using</td>
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<td>Positive Control (liquid)</td>
<td>1 x 1 ml bottle</td>
<td>+5°C (± 3°C)</td>
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<td>Negative Control (liquid)</td>
<td>1 x 1 ml bottle</td>
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<td>Monoclonal anti-VP7 peroxidase conjugate</td>
<td>1 x 4 ml bottle</td>
<td>+5°C (±3°C)</td>
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<td>• The diluted conjugate solution cannot be stored</td>
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<td>Revelation solution 2 (TMB) Ready to use</td>
<td>1 x 60 ml bottle</td>
<td>+5°C (±3°C)</td>
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<td>Stop Solution (H₂SO₄ 0,5M solution)</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (±3°C)</td>
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<td>• 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 ready to use when needed.</td>
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<td>• Identical for all the kits of the INSTITUT POURQUIER, it can be used equally in the different kits.</td>
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**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 sample 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 regulation in force, before discarding.

* The Institut Pourquier is at your disposal to supply the toxicity data sheets of the product.
**MATERIALS REQUIRED BUT NOT PROVIDED**

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 ≤ 5% for all the volumes indicated)
7) Disposable pipette tips
8) 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 ...).
9) Microplate covers (lid, aluminium foil or adhesive)

**USING INSTRUCTIONS FOR SAMPLES**

1) **DEPOSITING THE SERA**

Controls and samples are diluted at 1/5 using the following method (see note 1 and 2)

- Dispense:
  - 80 µl of "Dilution buffer 2" per well
  - 20 µl of undiluted positive control serum in A1
  - 20 µl of undiluted negative control serum in B1 and C1 (see notes 2 and 3)
  - 20 µl of each sample to test (one well per sample)

- Homogenize the contents of the wells by gently shaking the plate
- Cover the plate (with a lid, aluminium foil or adhesive)
- Incubate for **45 minutes (± 3 min) at 21°C (±5°C)**

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**Fig. 1: Distribution of the Samples**

**Notes:**

1) 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 96-well plates with U-shaped bottoms. It is therefore possible to transfer them rapidly (column by column) by using a multi-channel pipette. It is nonetheless essential to make the dilutions of the samples in the same way as for the controls.

2) The position of the controls in A1, B1 and C1 is not important, they may be dispensed anywhere on the plate. It may be better to add replicates of controls to the same plate in order to establish an average OD value. It is advised to place one positive and one negative control in the centre of the plate.
2) DEPOSITING THE CONJUGATE
   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 disappearance of the crystals which previously appeared at +21°C (± 5°C) as long as the whole 100 ml vial is used.
   b) Dilute this conjugate to 1/20 with the "Wash Solution".
   c) Dispense directly (without washing or emptying the wells) 100 µl of the diluted conjugate in each well.
   d) Cover the plate (with a lid or aluminium foil or adhesive) and leave to incubate for **45 minutes (± 3 min) at 21°C (±5°C)**.

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

**Notes:**
1) The care brought to the last washing is essential for a good implementation of the test.
2) If the washing is manual, the plate can be inverted and tapped dry on absorbent support, in order to completely empty the wells after the last washing.
3) If a lot of 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 without any modification on the validity of the results.

4) REVELATION
   a) Dispense 100 µl of "Revelation Solution 2" ready to use per well
   b) Leave to incubate for **10 minutes at +21°C (±5°C)** (away from the light).
   c) Add 100 µl of "stop solution" per well.
   d) Gently shake the plate until the coloured solution is homogenized. Wipe carefully the underside of the plate.

**Note:**
1. The 10-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 the water used, precision of the pipeting, 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 at 10 minutes ± 5 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.

5) READING
   a) Read the optical densities at 450 nm (OD.450). The photometer must first be blanked on air.
   b) Calculate for each sample the ratio between the OD of the sample and the OD of the negative control (S/N percentage). This value is obtained in the following manner:

\[
S/N \% : \frac{(OD \text{ 450 of analysed serum})}{(OD \text{ 450 of negative control})} \times 100.
\]
VALIDATION CRITERIA

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

- The negative control has a minimal mean OD 450 value of 0.700 and a maximal OD 450 value of 3.00 and
- The S/N percentage of the positive control is lower than 20%

INTERPRETATION

- Samples with S/N percentage equal or greater than 80% are considered coming from an animal, which does not carry specific antibodies to the BT virus.
- Samples with S/N percentage between 70% and 80% are considered as doubtful.
- Samples with S/N percentage equal or lower than 70% are considered coming from an animal, which carries specific antibodies to the BT virus.

Note:
For any sample showing S/N% in the doubtful zone, it is advised to confirm their status with another sample or another method.

<table>
<thead>
<tr>
<th>S/N percentage</th>
<th>INTERPRETATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 80%</td>
<td>Negative</td>
</tr>
<tr>
<td>70% - 80%</td>
<td>Doubtful</td>
</tr>
<tr>
<td>≤ 70%</td>
<td>Positive</td>
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LEGEND

 Modification in the using instructions

BIBLIOGRAPHY


