Bovine abortion

ELISA kit for serodiagnosis of
BoHV-4, Neosporose, Q Fever, Salmonellose and Leptospirose
Indirect test for blood sera and plasma
Diagnostic test for cattle

I - INTRODUCTION

The task of determining the cause of an abortion in cattle is generally a rather difficult one because, most of the time, it is the consequence of an event which happened weeks to months earlier. Often also, the foetus is maintained in the uterus for hours and even days after its death, and, when it is finally evacuated, it has undergone autolysis, in such a way that it is difficult to do any type of analysis. Also, many causes of abortion in cattle are to this day still unknown. Moreover, many pathogens are rarely looked for because they are difficult or dangerous to handle (*Coxiella burnetii*, *Chlamydia abortus* ..). Pathogens directly or indirectly responsible for abortions are numerous and varied, which complicates the diagnosis. Amongst the major pathogens responsible for abortions, one can find viruses (BoHV-1, BVDV, BoHV-4), bacteria (*Brucella abortus*, *Trueperella pyogenes*, *colibacille*, *streptocoque*, *Coxiella burnetii*, *Leptospira hardjo*, *Ureaplasma diversum*, *Campylobacter foetus*, *Borrelia coriaceae*, *Yersinia pseudotuberculosis*, *Chlamydia abortus*, *Salmonella*, *Listeria monocytogenes et Haemophilus somnus*), parasites (*Distoma hepatica*, *Trichomonas*, *Sarcocystis*, *Neospora*), fungi (*Aspergillus fumigatus*, *Mortierella wolfii*, as well as *Mucor*, *Absidia*, *Rhizopus*) and yeasts (*Candida*).
The kit aims at demonstrating the existence of a seroconversion toward the pathogenic agents mentioned above, in adult cattle, that it is, in the animal who aborted, but especially in the other animals of the herd, ideally in 10% of the livestock or the cowshed. Indeed, when the abortion occurs, the serological titer of the cow has often reached its maximum and it is not possible to show a seroconversion. It is thus preferable to test the other animals of the herd in order to verify whether the suspected infection is still active. If many animals show a clear seroconversion toward one of the five pathogens of the kit, one can attribute the abortion to this pathogen.

II – PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by a monoclonal antibody specific to *Neospora caninum* This antibody is used to trap *Neospora caninum* as well as to purify it from cultures of this protozoan. For *Salmonella dublin* and *Leptospira hardjo*, the plate sensitised by purified LipoPolySaccharide (LPS).
For BoHV-4 the plate sensitised by purified virus.
For *Coxiella burnetii* the plate sensitised by phase I and phase II antigenic extract from *Coxiella burnetii* cells.
The distribution of these pathogens on the microtitration plate is as follows:

Columns 1 & 7: BoHV-4
Columns 2 & 8: *Neospora caninum*
Columns 3 & 9: *Coxiella burnetii phase I + II*
Columns 4 & 10: *Salmonella dublin*
Columns 5 & 11: *Leptospira hardjo*
Columns 6 & 12: negative control

Columns 6 & 12 contain one monoclonal antibody. Using such a control reduces the number of false positives considerably. The test sera and plasma are diluted 1:100 in an appropriate buffer and incubated on the plate for one hour at 21°C +/- 3°C. The plate is washed and the conjugate, a peroxidase-labelled anti-bovine IgG1 monoclonal antibody, is added to the wells. The plate is reincubated at 21°C +/- 3°C for 1 hour. After this second incubation, the preparation is washed and the chromogen (tetramethylbenzidine) is added. This chromogen has the advantage of being more sensitive than the other peroxidase chromogens and not being carcinogenic. If specific immunoglobulins are present in the test sera the conjugate remains bound to the corresponding microwell and the enzyme catalyses the transformation of the colourless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the titre of specific antibody in the sample. The signals recorded for the negative control microwells are subtracted from the corresponding positive microwells. . It is possible to quantify the reactivity of an unknown sample on a scale ranging from 0 to +++++.

III - COMPOSITION OF THE KIT

- **Microplates**: 96-well microtitration plates. The distribution of the different valencies is indicated on the aluminium wrapper.
- **Washing solution**: One bottle of 20x concentrated washing solution. The solution crystallises spontaneously when cold. If only part of the solution is to be used, bring the bottle to 21°C +/- 3°C until disappearance of all crystals. Mix the solution well and remove the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water.
- **Dilution buffer**: One bottle of 5x colored, concentrated buffer for diluting the blood sera, plasma and conjugate. Dilute this concentrated dilution buffer 1:5 with distilled or demineralised water. If a deposit forms at the bottom of the container filter the solution on Whatman filter paper.
- **Conjugate**: One bottle of anti-bovine immunoglobulin-peroxidase conjugate (horseradish peroxidase-labelled anti-bovine IgG1 monoclonal antibody).
- **Positive serum**: One bottle of positive serum. Store this reagent between +2°C and +8°C.
- **Negative serum**: One bottle of negative serum. Store this reagent between +2°C and +8°C.
- **Single component TMB**: One bottle of the chromogen tetramethylbenzidine (TMB).
  Store between +2°C and +8°C protected from the light.
- **Stop solution**: One bottle of the 1 M phosphoric acid stop solution.

	BIO K 368/2
Microplates	2
Washing solution	1 X 100 ml (20 X)
Colored Dilution buffer	1 X 50 ml (5 X)
Conjugate	1 X 0,5 ml (50 X)
Positive serum	1 X 0,5 ml (1 X)
Negative serum	1 X 0,5 ml (1 X)
Monocomponent TMB	1 X 25 ml (1 X)
Stop solution	1 X 15 ml (1 X)

IV - ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

Distilled water, graduated cylinders, beakers, plastic tubes, tube rack, microplates for dilution, dispenser tips, reagent reservoir for multichannel pipettes, lid, adhesive for microplates, graduated automatic (mono- and multichannel) pipettes, microplate reader, and microplate washer and shaker (optional)

V - PRECAUTIONS FOR USE

- This test may be used for "in vitro" diagnosis only. It is strictly for veterinary use.
- The reagents must be kept between +2°C and +8°C. The reagents cannot be guaranteed if the shelf-life dates have expired or if they have not been kept under the conditions described in this insert.
- The concentrated wash solution and dilution buffer may be stored at room temperature. Once diluted, these solutions remain stable for six weeks if kept between +2°C and +8°C.
- Unused strips must be stored immediately in the aluminium envelope, taking care to keep the desiccant dry and the envelope's seal airtight. If these precautions are taken, the strips' activity can be conserved up to the kit's shelf-life date.
- Do not use reagents from other kits.
- The quality of the water used to prepare the various solutions is of the utmost importance. Do not use water that may contain oxidants (e.g., sodium hypochlorite) or heavy metal salts, as these substances can react with the chromogen.
- Discard all solutions contaminated with bacteria or fungi.
- The stop solution contains 1 M phosphoric acid. Handle it carefully.
- All materials and disposable equipment that come in contact with the samples must be considered potentially infectious and be disposed of in compliance with the legislation in force in the country.
- To guarantee the reliability of the results, one must follow the protocol to the letter. Special care must be taken in observing the incubation times and temperatures, as well as measuring the volumes and dilutions accurately.

VI – PROCEDURE

1- Bring all components to 21°C +/- 3°C before use. Remove the microplate from its wrapper.

2- DILUTION OF SAMPLES

2.1- Blood serum and plasma preparation

The blood serum and plasma samples must be diluted 1:100. Avoid using haemolysed samples or those containing coagula.

2.1.1- Dilution in tubes

Distribute 990 µl aliquots of dilution buffer, prepared as instructed in the section "Composition of the Kit", to 5 or 10 ml tubes. Add 10 µl aliquots of the samples to each of these tubes and mix briefly on a mechanical stirrer (final dilution: 1:100).

2.1.2- Dilution on a microplate

Distribute 20 µl aliquots of each of the samples to the microwells of a dilution plate. Add 180 µl of dilution buffer. Mix five times by pumping and surging or orbital agitation (dilution: 1:10). Distribute 90 µl aliquots of dilution buffer to the wells of the kit's microplate. Transfer 10 µl of the 1:10 prediluted samples. Mix five times by pumping and surging or orbital agitation (final dilution: 1:100).

2.2- Dilution of the kit's reference sera (positive and negative controls)

The positive and negative sera must be diluted 1:100. Do these dilutions in one step in a tube (see Point 2.1.1.) or in two steps on a dilution microplate (see Point 2.1.2.).

3- Distribute samples and the reference sera (100 µl/well) as follows: positive serum in wells H1 to H6, negative serum in wells G1 to G6, sample 1 in wells A1 to A6, sample 2 in wells B1 to B6 etc... Cover with a lid and incubate the plate at 21°C +/- 3°C for one hour.

4- Rinse the plate with the washing solution prepared as instructed in the section "Composition of the Kit". To do this, dispose of the microplate's contents by flipping it sharply over a container filled with an inactivating agent. Let the microplate drain upside-down on a sheet of clean absorbent paper so as to eliminate all liquid. Add 300 µl of the washing solution, and then empty the plate once again by flipping it over above the containment vessel. Repeat the entire operation two more times, taking particular care to avoid bubble formation in the wells. After these three rinses, go on to the next step. An automatic plate washer may also be used, but in this case take care that the needles do not get too close to the bottoms of the wells to prevent damaging the reagent layer.

5- Dilute the conjugate 1:50 in the buffer for dilution (for example, for one plate dilute 250 µl of the conjugate stock solution in 12.25 ml of diluent). Add 100 µl of the diluted conjugate solution to each well. Cover the plate with a lid and incubate the plate at 21°C +/- 3°C for one hour.
6- Wash the plate as described in step 4 above.
7- Add 100 µl of the chromogen solution to each well on the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution in the pipette has been contaminated.
8- Incubate for 10 minutes at 21°C +/- 3°C protected from the light and uncovered. This time is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
9- Add 50 µl of stop solution to each microwell. The blue colour will change into a yellow colour.
10- Read the optical densities in the microwells using a plate reader and a 450 nm filter. Results must be read fairly soon after the stopping solution has been added since the chromogen may crystallize in wells with strong signals and thereby distort the data.

VII – INTERPRETING THE RESULTS

Subtract from each value recorded in columns 1, 2, 3, 4, 5 the signal of the corresponding negative control well 6 and write down the result (calculation of Î OD. for the viral valencies). Allow for any negative values that may exist in performing this calculation. Carry out the same operations for the column corresponding to the positive and negative controls.

The test can be validated only if the positive control serum yields a difference in optical density at 10 minutes that is greater for each valence than:

BoHV-4	>	0.550
Neospora caninum	>	1.000
Coxiella burnetii	>	1.000
Salmonella dublin	>	1.000
Leptospira hardjo	>	1.000

And the negative control yields a difference in optical density at 10 minutes that is lower than 0,300.

Divide the signal read for each sample well by the corresponding positive control serum signal and multiply this result by 100 to express it as a percentage.

$$\text{Val(ue)} = \frac{\text{Delta OD Sample} * 100}{\text{Delta OD positive}}$$

Using the following table, determine each serum's degree of positivity.

	0		+		++		+++		++++		+++++
BoHV-4	Val <=	55 %	< Val <=	91 %	< Val <=	128 %	< Val <=	164 %	< Val <=	200 %	< Val
Neo.caninum	Val <=	12 %	< Val <=	40 %	< Val <=	69 %	< Val <=	97 %	< Val <=	125 %	< Val
Cox. burnetii	Val <=	43 %	< Val <=	64 %	< Val <=	84 %	< Val <=	105 %	< Val <=	125 %	< Val
Salm.dublin	Val <=	65 %	< Val <=	80 %	< Val <=	95 %	< Val <=	110 %	< Val <=	125 %	< Val
Lepto. hardjo	Val <=	16 %	< Val <=	37 %	< Val <=	58 %	< Val <=	79 %	< Val <=	100 %	< Val

A reliable diagnosis can be made only if frank seroconversion can be documented using two coupled serum samples taken at 2- to 3-week intervals. The first sample must be taken during the acute phase of the infection. A frank seroconversion is considered to have occurred if the signal increases by two orders of magnitude (for example, ++ -> ++++ or + -> +++). A sample must be considered positive if it yields a result that is greater than or equal to one plus sign (+).

VIII – ORDERING INFORMATION

Multiscreen AbELISA Abortion : 2 X 16 samples BIO K 368/2