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ANTIGENIC PULMOTEST FOR DETECTION OF *MYCOPLASMA BOVIS*

Sandwich ELISA test

Direct test for tissues lysats

Diagnosis test of *Mycoplasma Bovis* for bovines

I - INTRODUCTION

Mycoplasma bovis is associated with many cattle diseases, including arthritis, pneumonia in calves and young stock, mastitis, and genital infections. The infectious pneumonias that affect intensively-raised calves are responsible for sizable economic losses due to the mortality, treatment costs, and growth delays that they cause. These respiratory infections often involve multiple factors and are caused by interactions among viruses, mycoplasmas, and bacteria. Several species of Mycoplasma have been isolated from the respiratory tracts of calves. Some of them are most probably simple commensals or opportunistic species that merely worsen the lung damage caused by other agents. *Mycoplasma bovis* has been isolated from the lungs of calves with pneumonia. It is probably the most pathogenic species affecting livestock after *Mycoplasma mycoides mycoides. Mycoplasma bovis* can induce the development of pneumonia in gnotobiotic calves. *Mycoplasma bovis* is frequently found in association with *Mannheimia haemolytica* in pneumonia in calves.

II - PRINCIPLE OF THE TEST

As *Mycoplasma bovis* is usually present in diseased specimens in very small amounts, it must be enriched on an appropriate medium (Hayflick medium to which antibiotics and antimycotics have been added). After three days' growth at 37°C directly on the kit's microplates the cultures are tested for the presence of *Mycoplasma bovis.*

Rows A, C, E, and G on the 96-well microplates have been sensitised with a polyclonal antibody specific for *Mycoplasma bovis*. This antibody captures the mycoplasma as it grows in the culture medium. This immuno-enrichment technique increases the test's sensitivity significantly. The other rows on these microplates (rows B, D, F, and H) have been sensitised with a polyclonal antibody that is not specific for *Mycoplasma bovis*. This gives us a true negative control to determine whether a specific binding reaction has taken place. Using such a control reduces the number of false positive specimens considerably.

After the three-day culture step the preparation is washed and the conjugate, which is an anti-*Mycoplasma bovis* specific monoclonal antibody coupled to peroxidase, is added. After one hour's incubation at 21°C +/- 3°C and a second washing step the chromogen (TMB) is added. This chromogen has the advantages of being more sensitive to peroxidase than the other chromogens and not being carcinogenic. If *Mycoplasma bovis* is present in the culture, the conjugate remains fixed to the well containing the mycoplasma antigen and the enzyme catalyses the colourless chromogen's transformation into a blue compound. The intensity of the blue colouring is proportionate to the sample's *Mycoplasma bovis* titre. The signal recorded for the negative well sensitised with the control polyclonal antibody is subtracted from the signal read for the positive well sensitised with anti-*Mycoplasma bovis* specific polyclonal antibody. A control antigen is provided with the kit to validate the results. This control antigen consists of a lyophilised inactivated *Mycoplasma bovis* culture.

III - COMPOSITION OF THE KIT

- **Microplates**: Two 96-well microtitration plates. Rows A, C, E, and G are sensitised by anti-*Mycoplasma bovis* specific polyclonal antibody, while rows B, D, F, and H are sensitised by the control antibody (polyclonal antibody not specific for *Mycoplasma bovis*).
- **Washing solution**: One 100-ml 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 withdraw the necessary volume. Dilute the buffer 20-fold with distilled or demineralised water.
- **Conjugate**: one 25ml vial of coloured conjugate. **This solution is ready to use.**
- **Control antigen**: 2 vials containing control antigen. Reconstitute this antigen with 0.5 ml of distilled or demineralised water. The reconstituted reagent may be kept at -20°C. Divide the reconstituted antigen into two portions before freezing in order to avoid repeated freezing and thawing. If these precautions are taken the reagent may be kept for several months.
- **Single component TMB** One 25-ml bottle of the chromogen tetramethylbenzidine (TMB). Store between +2°C and +8°C protected from light. **This solution is ready to use.**
- **Stopping solution**: One 15-ml bottle of the 1 *M* phosphoric acid stop solution.
- **Hayflick medium**: One 30-ml bottle of sterile medium.
- **Antibiotic-antimycotic mixture**: 2 vials containing a mixture of freeze-dried antibiotics and antimycotics.

	BIO K 341/2
Microplates	2
Washing solution	1 X 100 ml (20 X)
Conjugate	1 X 25 ml (1 X)
Control antigen	2 X 0.5 ml (1 X) (freeze-dried)
Single component TMB	1 X 25 ml (1 X)
Stopping solution	1 X 15 ml (1 X)
Hayflick medium	1 X 30 ml
Antibiotic-antimycotic mixture	2 (freeze-dried)

IV - ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

Distilled water, sterile PBS, sterile dilution microplate, pair of scissors, Minigrip® bag, graduated cylinders, beakers, plastic tubes, tube rack, 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 may be stored at room temperature. Once diluted, this solution remains 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

Remark: First, the samples must be diluted (four sequential 9-fold dilutions per sample), because the growth of contaminant micro-organisms at low dilutions can hobble the growth of *Mycoplasma bovis*. The proposed dilution range (1:9 to 1:6561) is designed to cover a range of concentrations compatible with the number of *Mycoplasma bovis* that one is likely to find in diseased specimens from cattle. Second, one must allow for two wells for each dilution, because the kit has a twin-well design pairing a test well with a control well.

CULTURING THE SPECIMENS

1- Prepare the enrichment medium under sterile conditions. Suspend the contents of one vial of the antibiotic-antimycotic mixture in 1 ml of enrichment medium. Dilute the antibiotic-antimycotic solution in 12 ml of Hayflick medium. The resulting medium is ready for use. It is stable for only a short while (3 days between +2°C and +8°C) because the antibiotics and antimycotics are unstable in liquid solution. Hayflick medium contains foetal calf serum. Its stability is limited to 6 months. Additional vials of the antibiotic-antimycotic mixture can be ordered if the kit is used in several stages.

2- Enrichment is possible from various types of specimen (lung tissue homogenate, bronchio-alveolar washings, nasal swabs, joint needle biopsy fluid, milk, lochia, etc.). The samples must be sent to the laboratory as quickly as possible, preferably between +2°C and +8°C, and in a storage medium that does not contain any antibiotics or substances likely to inhibit the growth of *Mycoplasma bovis*. To prepare the lung tissue homogenate, work as follows: Take an approximately 1 cm3 lung fragment, place it in 10 ml of sterile PBS solution in a Petri dish, and cut it up into very small pieces with a pair of scissors. . This equipment and all reagents must be sterilised before and after use to avoid contaminating samples. Lung tissue may be stored in a freezer. Tissue homogenates may not be frozen, for this decreases *Mycoplasma*'s viability greatly.

3- Use a sterile microplate to make up the four dilutions of each sample. Using a multichannel pipette with sterile microtips and working under sterile conditions, distribute complete Hayflick medium in all of the wells of the strips used to test the samples at the rate of 240 µl per well. Allow four microwells per sample. Work under a laminar flow hood or next to a flame using sterile procedures. Add 30 µl of the first sample sterilely to the 240 µl of medium in the first microwell (A1) by means of 2-3 passes of the microtip (1:9 dilution). See the diagram. Proceed in the same way for the other samples (Sample 2 in A2, Sample 3, in A3, and so on). Using a multichannel pipette, transfer 30 µl from the first row of dilutions to the well in row B (1:81 dilution). Proceed in the same way for the wells in Row C (1:729 dilution) and then in Row D (1:6561 dilution).

4- Take the microplate provided in the kit out of its wrapper and break off the number of strips necessary to test the samples. Count out an additional strip for the controls. Using a multichannel pipette and starting from the highest dilution (Row D), transfer 100 µl from each well in Row D to the wells in Rows H and G of the ELISA microplate (see the diagram). Proceed in the same way for the other rows (see diagram). For the control strip, distribute 100 µl of the positive control per well to wells A1 and B1. Distribute 100 µl of Hayflick solution per well to wells E1 and F1.

5- Incubate the ELISA microplate at 37°C in a Minigrip® bag with a piece of paper soaked in water (to keep the medium from drying out) for three days. Use a lid to protect the plate.

6- After the three-day enrichment step, rinse the plate with the washing solution prepared as instructed in the 'Composition of the Kit' section. To do this, empty the microplate of its contents by flipping it over suddenly over a sufficiently large vessel containing a sodium hypochlorite solution. Tap the upside-down microplate against a sheet of clean absorbent paper so as to remove all liquid. Fill the used wells with a washing solution either by means of a spray bottle or by plunging the microplate in a suitably-dimensioned container, then empty the plate once again by flipping it over a sink. Repeat the entire operation two more times, taking special care to avoid the formation of bubbles in the wells. Upon completing these three washes, go on to the next step.

7- Distribute the conjugate solution at the rate of 100 µl per well. Incubate at 21°C +/- 3°C for 1 hour with a lid.

8- Wash the plate as described in Step6.

9- 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. Incubate at 21°C +/- 3°C and away from light for 10 minutes without lid. This time interval is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.

10- Add 50 µl of stop solution to each well.

11- Read the optical densities by means of a microplate spectrophotometer with a 450 nm filter. The results must be read as quickly as possible after the stop solution has been applied, for in the case of a strong signal the chromogen can crystallise and lead to incorrect measurements

VII – INTERPRETING THE RESULTS

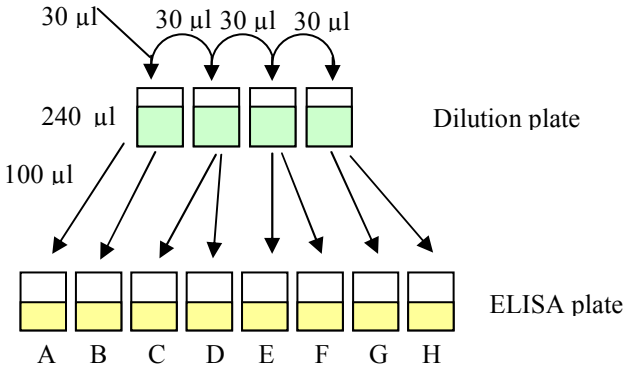
Calculate the net optical density of each sample by subtracting from the reading for each sample well the optical density of the corresponding negative control.
Proceed in the same way for the positive control antigen.
The test is validated only if the positive control antigen yields a difference in the optical density at 10 minutes that is greater than the value given on the QC data sheet.

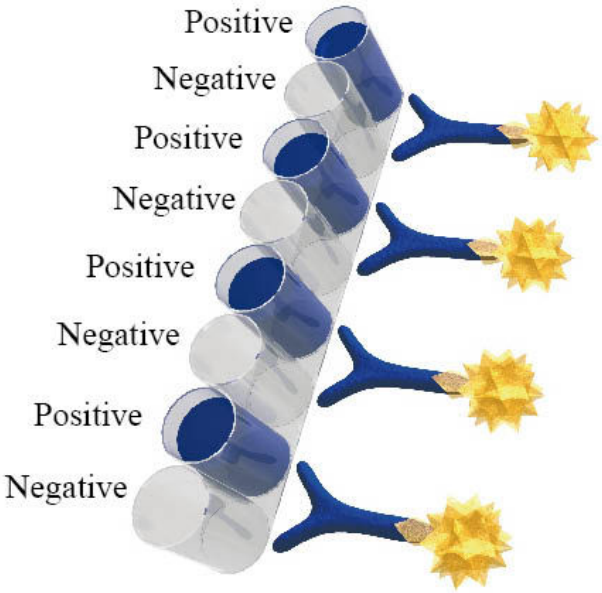
Divide the signal read for each sample well by the corresponding positive control signal and multiply this result by 100 to express it as a percentage. Using the first table in the quality control procedure, determine each sample's status (positive, negative).

$$Val = \frac{\text{Delta DO spl} * 100}{\text{Delta DO pos}}$$

VIII – ORDERING INFORMATION

BIO-X PULMOTEST *MYCOPLASMA BOVIS:* 2 X 12 tests BIO K 341/2




Positive
Negative
Positive
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