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Mannheimia haemolytica

ELISA Kit for serodiagnosis of *Mannheimia haemolytica*
Indirect test for blood sera and plasma
Diagnostic test for cattle
Monowell

I - INTRODUCTION

Mannheimia haemolytica is the cause of epizootic pneumonia in cattle known as Shipping Fever or pneumonic *pasteurellosis*. 90% is caused by *Mannheimia haemolytica* Biotype A, serotype 1 but also *Pasteurella multocida*. It is usually secondary to viral infections such as parainfluenza - 3 or IBR, bacterial infections such as *Mycoplasma* or environmental stress.
Mannheimia haemolytica may contribute to Enzootic pneumonia of calves; Enzootic pneumonia of lambs and peritonitis in sheep. It also causes gangrenous mastitis in sheep.
Mannheimia strains often produce a cytotoxin, known as leukotoxin, which kills leukocytes of ruminants. Leukotoxin is a member of the RTX group toxins, and is probably largely responsible for the pathogenicity of the bacteria in septicaemia and pneumonia.

II – PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by purified LipoPolySaccharide (LPS) from *Mannheimia haemolytica*. The entire surface of each microplate has been sensitised with LPS.
The test blood sera and plasma are diluted in the dilution buffer. The plate is incubated and washed, then the conjugate, protein G peroxidase-labelled, is added to the wells.
The plate is incubated a second time at 21°C +/- 3°C. After the second incubation, the plate is washed again and the chromogen (tetramethylbenzidine) is added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic. If specific *Mannheimia haemolytica* immunoglobulins are present in the test sera or plasma the conjugate remains bound to the microwell that contains the bacterial recombinant antigen and the enzyme catalyses the transformation of the colorless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the titre of specific antibody in the sample.

III - COMPOSITION OF THE KIT

- Microplates: 96-well microtitration plates (12 strips of 8 wells). The entire surface of each microplate has been sensitised with LPS from Mannheimia haemolytica.
- 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 all crystals have

disappeared. 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 and concentrated buffer for diluting the blood sera, plasma and conjugate. The bottle's content is to be diluted with distilled or demineralised water. If a deposit forms at the bottom of the receptacle filter the solution on Whatman filter paper.
- Conjugate: One bottle Protein G, horseradish peroxidase-labelled.
- Positive reference: One bottle of positive serum. Store this reagent between +2°C and +8°C.
- Negative reference: 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 light. This solution is ready to use.
- Stop solution: One bottle of the 1 M phosphoric acid stop solution.

	BIO K 139/2
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)
Single component 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 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 sera and plasma preparation
The blood serum and plasma samples must be diluted 1:100. Avoid using haemolysed samples or those containing coagulum.

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 the samples (blood serum, plasma) at the rate of 100 µl per well. One well per sample.
Proceed in the same manner for the reference sera (positive and negative sera). Cover with a lid and incubate the plate at 21°± 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, eliminate 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 particular care must be taken to avoid any contact between the needles and the bottom of the wells to prevent any damage of the reagent layer.
5- Dilute the conjugate 1:50 in the dilution buffer (for example, for one plate dilute 250 µl of the conjugate stock solution in 12.250 ml of diluent). Add 100 µl of the dilute conjugate solution to each well. Cover with a lid and incubate the plate at 21°± 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 per 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 filtre. Results must be read fairly soon after the stopping solution has been added since the chromogen may cristallize in wells with strong signals and thereby distort the data.

VII – INTERPRETING THE RESULTS

The test can be **validated** only if the difference between the optical density readings of the positive control serum and negative control serum (OD positive serum - OD negative serum) at ten minutes is greater than 0,400 and the negative serum yields an optical density that is lower than 0,300.

Calculate each serum's coefficient by means of the following formula:

$$\text{Sample's Coeff.} = \frac{\text{OD sample - OD negative serum}}{\text{OD positive serum - OD negative serum}} \times 100$$

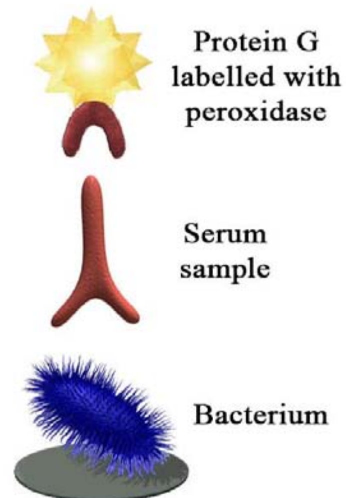
Using the following tables, determine each serum's or plasma's degree of positivity.

0		+		++		+++		++++		+++++
Val <=	23 %	< Val <=	87 %	< Val <=	151 %	< Val <=	216 %	< Val <=	280 %	< 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 (two plusses; 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

Monoscreen AbELISA *Mannheimia haemolytica* 2x96 tests BIO K 139/2



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