MONOSCREEN Ab ELISA

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Fasciola hepatica

ELISA Kit for serodiagnosis of Fasciolosis
Indirect test for blood sera, plasma and milk
Diagnostic test for cattle and sheep
Double wells

I - INTRODUCTION

Bovine fasciolosis caused by the digenic trematode *Fasciola hepatica* is a worldwide parasitic disease common in ruminants. This two-host life cycle parasite is classically found in farms where all conditions for the survival and the multiplication of the snail intermediate host *(Galba truncatula)* are fulfilled. This snail is mainly found in damp meadows (watering-places, brooks, springs).
Fasciola egg shedding occurs with faeces. Hatching follows in water and gives rise to the miracidium which infests the snail. After multiplication in this host, cercariae are eliminated and give rise to infectious metacercariae fixed on a plant holder.
Once ingested by a ruminant, young flukes migrate through the liver to reach bile ducts. The prepatent period is 8 to 10 weeks. Adults appear in the bile ducts and start to lay eggs.
Liver damage and acute disease (especially in sheep) are caused by migrating immature parasites. Chronic disease occurs in cattle during the biliary phase.
Zootechnical characteristics are hampered by the disease. Decrease in milk yield (-10%), weight loss, intermittent diarrhoea, anemia and fertility problems.
Diagnosis of *Fasciola hepatica* in cattle can only be made after 8 to 10 weeks by coprological examination of faecal material. However, sometimes even repeated fecal examination cannot indentify any *Fasciola hepatica* infection due to the lack of sensitivity of this method.
Acute distomatosis of the sheep is characterized by anemia and sometimes sudden mortality and chronic distomatosis by anemia, reduction of the dairy production, reduction of the average daily profit and oedemas

II – PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by a monoclonal antibody specific to one protein of *Fasciola hepatica*. This antibody is used to trap the protein as well as to purify it from lysate of the parasite. The plate's odd columns (1, 3, 5, 7, 9 and 11) contain the specific protein, whereas the even columns (2, 4, 6, 8, 10 and 12) contain only the monoclonal antibody. This is a genuine negative control to differentiate specific anti-*Fasciola hepatica* antibodies from non specific ones.
The test blood sera, plasma or milks are diluted in the dilution buffer. The plate is incubated and washed, then the conjugate, a peroxidase-labelled anti-ruminant IgG1 monoclonal antibody, is added to the wells. The plate is then incubated a second time at 21°C+/- 3°C, washed again and the chromogen tetramethylbenzidine (TMB) is

added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic. If specific *Fasciola hepatica* immunoglobulins are present in the test sera or in milk the conjugate remains bound to the microwell that contains the 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 titer of specific antibody in the sample. The signal read off the negative control microwell is subtracted from that of the positive microwell sensitised by the antigen. The interpretation of the results is done by comparing the signals of the samples (serum, plasma or milk) with those of the positive controls.
Kit allows the analysis of pool of 10 individual sera or plasma. It allows too an analysis of bulk tank milk.

III - COMPOSITION OF THE KIT

- **Microplates**: 96-well microtitration plates (6 strips of 16 wells). The odd columns (1, 3, 5, 7, 9, 11) are sensitised by the *Fasciola hepatica* antigen captured by the monoclonal antibody and the even columns (2, 4, 6, 8, 10, 12) contain only the monoclonal antibody.
- **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, concentrated buffer for diluting the blood sera, plasma, milks 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**: 1 bottle of anti-bovine immunoglobulin-peroxidase conjugate (horseradish peroxidase-labelled anti-bovine IgG1 monoclonal antibody).
- **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.
- **Tracer:** One bottle of tracer.The tracer is a reference sample that can be used to check the intra-laboratory reproducibility of the kit's batch.
  Intra-laboratory reproducibility: Degree of agreement between the results of reiterated tests on the same sample with an identical technical protocol in a given laboratory under variable working conditions.
- **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 211/2	BIO K 211/5
Microplates	2	5
Washing solution	1 X 100 ml (20 X)	1 x 250 ml (20x)
Colored Dilution buffer	1 X 50 ml (5 X)	1 x 100 ml (5 X)
Conjugate	1 X 0.5 ml (50 X)	1 X 1.4 ml (50 X)
Positive serum	1 X 0.5 ml (1 X)	1 X 0.5 ml (1 X)
Negative serum	1 X 0.5 ml (1 X)	1 X 0.5 ml (1 X)
Tracer	1 X 0.5 ml (1 X)	1 X 0.5 ml (1 X)
Single component TMB	1 X 25 ml (1 X)	1 x 55 ml (1 X)
Stop solution	1 X 15 ml (1 X)	1 x 30 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 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) and tracer.
The positive and negative sera and the tracer 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.).

2.3-Milk preparation
Centrifuge at 4000 g for 20 minutes. Take up the middle layer of liquid by means of a glass Pasteur pipette inserted through the upper layer of cream, taking care not to touch the underlying cell sediment.
The milk samples must be diluted 1:4.

2.3.1- Dilution in tubes
Distribute 750 µl aliquots of dilution buffer, prepared as instructed in the section "Composition of the Kit", to 5 or 10 ml tubes. Add 250 µl aliquots of milk to each of these tubes and mix briefly on a mechanical stirrer (final dilution: 1:4).

2.3.2- Dilution on a microplate
Distribute 60 µl of each sample to the microwells of a dilution plate. Add 180 µl of dilution buffer. Mix five times by pumping and surging or orbital agitation (dilution: 1:4). Transfer 100 µl aliquots of the diluted samples to the kit's microplate.

3- Distribute the samples (blood serum, plasma, or milk) at the rate of 100 µl per well. For example, the following pattern may be followed: Positive serum in wells A1 and A2, Negative serum in wells B1 and B2, tracer in wells C1 and C2, Sample 1 in wells D1 and D2, and so on. 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.
   Using a plate washer (whether automatic or manual) is also recommended. However, the depth of the needles’ immersion must be set so as not to disturb the layer of reagents adsorbed to the bottom of each well.
   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

Subtract from each value recorded for the odd columns the signal of the corresponding negative control well and write down the result. In performing this calculation, allow for any negative values that may exist. Carry out the same operations for the column corresponding to the positive and negative controls.
The test can be **validated** only if the positive serum yields a difference in optical density at 10 minutes that is greater than 0,800 and the negative serum yields a difference in optical density 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, plasma’s or milk’s degree of positivity.

	0		+/-		+		++		+++
Individual sample	Val	<10%<=	Val	<15%<=	Val	<45%<=	Val	<75<=	Val
Pool of 10 samples	Val	<5%<=	Val	<15%<=	Val	<45%<=	Val	<75<=	Val

The degree of positivity is interpreted as follows:

0: No *Fasciola hepatica* infestation
+/-: Dubious outcome. Redo the test in a month.
+: Low-grade infestation
++: Moderate infestation
+++: Heavy infestation

These levels may be used to interpret the degree of infestation in an individual animal or the flock/herd. It is important, however, that the farm’s veterinarian determines the serological status of the animal or flock/herd taking the following parameters into account:

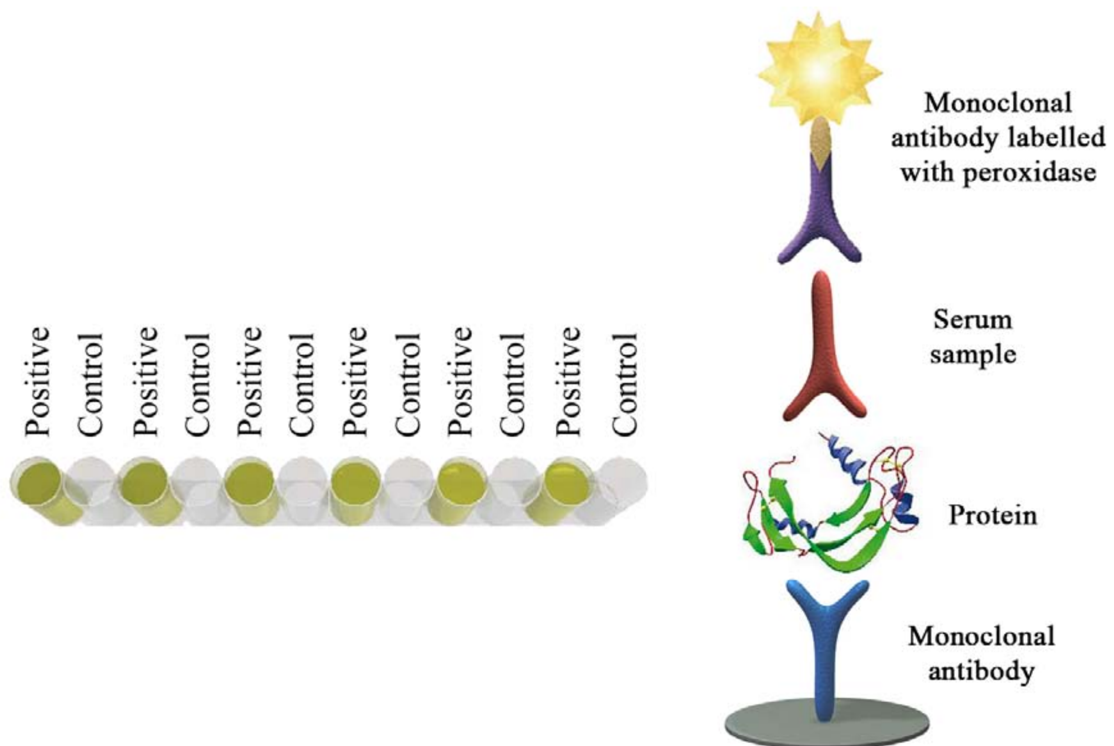
- time of year (stabling or pasturing);
- stock structure (groups of animals with and without grazing experience);
- fluke treatments already administered; and
- weather or environmental conditions (pond snail-infested sites).

For example, it is well established that strongly positive animals' serum titres will regress very gradually after they are treated for flukes. The strengths of their reactions can thus drop from +++ to + or even turn negative over several months, provided that they are protected from all new infestations.

The *Fasciola hepatica* ELISA kit can be used to analyse mixed milk samples (tank milk).

VIII – ORDERING INFORMATION

Monoscreen AbELISA *Fasciola hepatica*	2x48 tests	BIO K 211/2
	5x48 tests	BIO K 211/5



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