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For Veterinary Use Only

**THIS KIT IS INTENDED FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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Indirect test for blood sera, plasma and milk.

ELISA KIT FOR SERODIAGNOSIS OF FASCIOLA HEPATICA IN BOVINE, or OVINE (double wells).

1 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 (*Lymnea truncata*) 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 identify 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.

2 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 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 enzyme's substrate (hydrogen peroxide) and the chromogen tetramethylbenzidine (TMB) are 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 or milk) with those of the positive controls.

3 COMPOSITION OF THE KIT

1. **Microplates** : Two 96-well microtitration plates. 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.
2. **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 remove the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water. Store the diluted solution at 4°C.
3. **Dilution buffer** : One 50 ml bottle of 5x concentrated buffer for diluting the blood sera, milks and conjugate.
The bottle's contents is to be diluted with distilled or demineralised water. This solution will keep at 4°C for at least 3 month. If a deposit forms at the bottom of the receptacle filter the solution on Whatman filter paper.
4. **Conjugate**: One bottle 0.5 ml, 50X, of anti-bovine immunoglobulin-peroxidase conjugate (horseradish peroxidase-labelled anti-bovine IgG1 monoclonal antibody).
5. **Positive reference serum** : One bottle of positive serum.
Reconstitute this serum with 0.5 ml of distilled or demineralised water. The reconstituted serum may be kept at -20°C. Divide the reconstituted serum into several portions before freezing in order to avoid repeated freezing and thawing. If these precautions are taken the reagent may be kept for several months.
6. **Chromogen solution** : One 2 ml drop-dispenser bottle of the chromogen tetramethylbenzidine.
Store at 4°C.
7. **Substrate solution** : One 30 ml bottle of the hydrogen peroxide substrate solution.
Store this reagent at 4°C.
8. **Stop solution** : One 15 ml bottle of the 1 M phosphoric acid stop solution.

4 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 4 and 8°C. The positive serum must be kept at -20°C once it is reconstituted. The reagents cannot be guaranteed if the shelf-life dates have expired or they have not been kept under the conditions described in this insert.

Do not use reagents from other kits.

The quality of the water used to make up 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 carefully.

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5 PROCEDURE

1. Bring all reagents at 21°C +/- 3°C at least 30 minutes before starting the test.
2. Remove the microplate from its wrapper.
3. If the test is realized with **serum samples**, place 1 ml aliquots of the dilution solution, prepared as instructed in the "Composition of the Kit" section, in 5- or 10-ml hemolysis tubes.
Add 10 µl of the serum samples to each of these tubes and shake briefly on a mechanical agitator.
Proceed in the same manner for the positive serum.
4. Add **100 µl** aliquots of the **1:100 diluted serum samples** to the wells as follows:
positive reference serum: wells A1 and A2, sample 1 in wells B1 and B2, sample 2 in wells C1 and C2, etc...
5. For serum samples, incubate the plate at 21°C +/- 3°C for one hour.
6. For **milk**, add **directly** on the plate **75 µl of dilution solution per well and 25 µl of milk** (shake briefly on a plate agitator).
Example of distribution of the samples: positive reference serum: wells A1 and A2, sample 1 in wells B1 and B2, sample 2 in wells C1 and C2, etc...
7. For milk samples, incubate the plate at 21°C +/- 3°C for one hour. It is also possible to incubate plate overnight at 4°C to get higher signals.
8. **Rinse the plate** with the washing solution, prepared as instructed in the "Composition of the Kit" section, as follows: empty the microplate of its contents by flipping it over sharply above a sink. Tap the microplate upside down against a piece of clean absorbent paper to remove all the liquid. Fill the used wells with the washing solution using a squeeze bottle or by plunging the plate in a vessel of the right dimensions, then empty the wells once more by turning the plate over above a sink. Repeat the entire operation two more times, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed **three times** 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.
9. Dilute the conjugate 1:50 in the dilution buffer
(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.
Incubate for 1 hour at 21°C +/- 3°C.
10. Wash the plate as described in step 8 above.
11. Prepare the indicator solution extemporaneously as follows:
add 500 µl (12 drops) of chromogen to 9.5 ml of the substrate solution. Mix thoroughly, then apply to the plate immediately in volumes of **100 µl** per microwell.
At the time of distribution of the chromogen-substrate mixture on the plates the solution must be completely colourless. If a blue colour appears at this stage, the solution is contaminated with peroxidase. If this occurs, the chromogen-substrate solution must be discarded and a new solution made up using absolutely clean glassware and equipment.
12. Incubate for 10 minutes at 21°C +/- 3°C. This time is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
13. Add **50 µl of stop solution** per microwell.
14. 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.

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For Veterinary Use Only**6 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 control. The test can be validated only if the positive control yields a difference in optical density at 10 minutes that is greater than the value given in the QC data sheet:

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.

Using the first table in the quality control procedure, determine each serum or milk status.

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 determine 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).