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 (Lymnaea 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 infects 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. One of the main problems in the control of this disease is the lack of sensitive and convenient tests for the diagnosis of Fasciola spp. infection in large herds under field conditions, and for monitoring the efficacy of flukicide treatments. Diagnosis of animal fascioliasis is largely based on microscopic demonstration of parasite ova in feces, but Fasciola spp. egg shedding is intermittent and irregular. Serological techniques allow the detection of specific antiparasite circulating antibodies but tests of this type are of limited diagnostic value in endemic areas because antibody titers remain at high levels even when animals have been successfully treated. BIO K 201 is a reliable and ultrasensitive method for detecting subnanogram amounts of F. hepatica antigens in feces from sheep and cattle, facilitating early diagnosis.

**Reliable Results**
The use of monoclonal antibody as conjugate ensures excellent specificity and very reliable results. The use of polyclonal antibody as capture reagent ensures very high sensitivity.

**Early diagnosis.** Allows monitoring of flukicide treatments (negative results 1-3 weeks after treatment)

**Ease-of-Use**
Minimal hands-on-time
Room temperature incubation
Results available in 200 minutes for single or batch testing

**Flexibility**
Results can be read visually or spectrophotometrically.

**EIA Procedure**
1. Microplate coated with polyclonal antibody
2. Add samples and positive control.
   Incubate 1 hour at 21°C +/- 3°C
   Wash
3. Add first conjugate (monoclonal biotin conjugated).
   Incubate 1 hour at 21°C +/- 3°C .
   Wash
4. Add second conjugate (avidin-peroxidase).
   Incubate 1 hour at 21°C +/- 3°C.
   Wash
5. Add chromogen + Substrate
   Wait 10 minutes.
Example of results

A group of 21 four-months old lambs was experimentally infected with variable number of metacercariae (5 to 40) obtained from experimentally infected Lymnaea truncatula snails. Six lambs infected with 40 metacercariae were treated with a single oral dose of 10 mg/kg of triclabendazole (Fasinex 5%) at 14 weeks post infection. Fecal samples were taken from each animal just before infection and then weekly for 18 weeks post infection. At the end of the experiment, all animals were necropsied and all liver parasites collected (Mezo et al. 2004).

### Untreated animals

<table>
<thead>
<tr>
<th>Weeks post infection</th>
<th>14 weeks</th>
<th>15 weeks</th>
<th>16 weeks</th>
<th>17 weeks</th>
<th>18 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean E.P.G. ±SD</td>
<td>56 ± 73 (15/15)</td>
<td>64 ± 93 (15/15)</td>
<td>59 ± 92 (15/15)</td>
<td>95 ± 115 (15/15)</td>
<td>100 ± 102 (15/15)</td>
</tr>
<tr>
<td>Mean O.D. ±SD</td>
<td>2.042 ± 0.189 (15/15)</td>
<td>1.981 ± 0.298 (15/15)</td>
<td>2.135 ± 0.105 (15/15)</td>
<td>2.005 ± 0.275 (15/15)</td>
<td>2.139 ± 0.224 (15/15)</td>
</tr>
<tr>
<td>Range fluke burden in the liver</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1-36 (15)</td>
</tr>
</tbody>
</table>

### Treated animals

<table>
<thead>
<tr>
<th>Weeks post infection</th>
<th>14 weeks</th>
<th>15 weeks</th>
<th>16 weeks</th>
<th>17 weeks</th>
<th>18 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean E.P.G. ±SD</td>
<td>67 ± 65 (5/6)</td>
<td>0 (0/6)</td>
<td>5 ± 3 (5/6)</td>
<td>12 ± 13 (2/6)</td>
<td>45 ± 59 (2/6)</td>
</tr>
<tr>
<td>Mean O.D. ±SD</td>
<td>1.873 ± 0.294 (6/6)</td>
<td>0.530 ± 0.06 (1/6)</td>
<td>0.13 ± 0.025 (1/6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Range fluke burden in the liver</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

(*): Mean O.D. values and mean E.P.G. counts were calculated using data from positive animals only.

Percentages of experimentally infected lambs testing positive by ELISA and by fecal examination for eggs, at different times after infection (Mezo et al. 2004).
Mean optical density values obtained in ELISA of fecal supernatants from lambs experimentally infected with metacercariae (n = 15) or non infected (n = 6). (Mezo et al. 2004).

Fasciola hepatica coproantigen concentrations measured by ELISA in fecal supernatants from 180 cows killed at the slaughterhouse. The dashed line is the detection limit in the ELISA assay (0.6 ng/ml, corresponding to the lowest concentration giving an OD reading 0.15 higher than the cutoff. (Mezo et al. 2004).
Four cows were infected with 300 metacercariae at week 0. They were treated with Triclabendazole at week 14. Optical densities were followed at week 14 and week 15. None of these treated animals had flukes in the liver at the slaughterhouse.

## Conclusions

The Fasciola Antigen ELISA detection kit (BIO K 201) is a reliable, robust and ultrasensitive method for sheep and cattle, capable of detecting subnanogram amounts of specific excretory-secretory antigens in feces. Diagnosis of animal fascioliasis is largely based on microscopic detecting Fasciola hepatica infections in demonstration of parasite ova in feces, but Fasciola spp. Egg shedding is intermittent and irregular and does not begin until 10-12 weeks postinfection or later (Zimmerman et al., 1982). Besides its low sensitivity, which can result in underdiagnosis of weak infections (Conceição et al., 2002), coproscopy is a laborious technique requiring each sample to be examined individually by training personnel so that it is not suitable for analysing large herds. Immunological techniques using selected excretory-secretory antigens from Fasciola spp. Allow the detection of specific antiparasite circulating antibodies within 3-5 weeks of the infection (Mezo et al., 2003) and can readily be automated. Nevertheless, tests of this type are of limited diagnostic value in endemic areas because antibody titers remain at high levels even when animals have been successfully treated (Sanchez-Andrade et al., 2001)

## Literature cited


## BIO K 201 FASCIOLA HEPATICA ANTIGEN ELISA

<table>
<thead>
<tr>
<th></th>
<th>Cow 1</th>
<th>Cow 2</th>
<th>Cow 3</th>
<th>Cow 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triclabendazole</strong></td>
<td>Week 14: 2.320</td>
<td>Week 15: 0.019</td>
<td>Week 14: 2.530</td>
<td>Week 15: 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Week 14: 2.150</td>
<td>Week 15: 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Week 14: 0.940</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Week 15: 0.05</td>
</tr>
</tbody>
</table>

2 plates of 12 strips for a total of 96 tests
2 vials of positive control
1 vial of conjugate I
1 vial of conjugate II
1 bottle of washing solution
1 bottle of dilution buffer
1 drop-dispenser bottle of chromogen
1 bottle of substrate solution
1 bottle of stopping solution
1 year of stability at 4°C
I - INTRODUCTION

Bovine fascioliasis caused by the digenic trematode Fasciola hepatica (common liver fluke) is a worldwide parasitic disease common in ruminants. This two-host parasite is classically found on farms where all conditions for the survival and reproduction of its intermediate host, the snail Lymnea truncata, are fulfilled. This snail is found mainly in damp meadows (watering places, brooks, springs, etc.). Fasciola eggs are shed in the faeces. Hatching follows in water and gives rise to miracidia, which infest the snail. After multiplication in this host, cercariae are eliminated and encyst on aquatic plants as infectious metacercariae.

Once ingested by a ruminant, young flukes migrate through the liver to reach the 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.

The disease depresses the animals’ development and productivity, causing decreases in milk yields (-10%), weight loss, intermittent diarrhoea, anaemia and fertility problems.

Diagnosis of Fasciola hepatica in cattle can be made only after 8 to 10 weeks by coprological examination of faecal material. However, sometimes even repeated faecal examinations will fail to identify a Fasciola hepatica infection due to the method’s lack of sensitivity.

Bio-X Diagnostics’ antigenic ELISA kit allows the detection of coproantigens in infested cattle’s faecal material. These coproantigens can be found in the faeces even outside the fluke’s egg-laying period. Unlike serotests, the samples will test positive with the antigenic assay only if flukes are present in the bile ducts.

II - PRINCIPLE OF THE TEST

Rows A, C, E, and G of the 96-well microplate have been sensitised with a specific polyclonal antibody against Fasciola hepatica. This antibody captures the coproantigens in the faecal material. The other rows on the microplate (rows B, D, F, and H) have been sensitised with a polyclonal antibody that is not specific for the parasite. These control rows allow differentiation between a specific immunological reaction and nonspecific binding so as to eliminate false positives.
The faecal material is diluted in dilution buffer and incubated on the microplate for 1 hour at room temperature. After this first incubation step, the plate is washed and incubated for 1 hour with the first conjugate (a specific monoclonal antibody against an antigenic determinant of Fasciola hepatica coupled to biotin), then the plate is incubated at room temperature for 1 hour. The plate is then washed, the second conjugate – a peroxidase-coupled avidine specific to biotin – is applied, and the plate is incubated at room temperature for another hour. After this second incubation, the plate is washed again and the enzyme substrate (hydrogen peroxide) and the chromogen (tetramethylbenzidine) are added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic.

If Fasciola hepatica coproantigens are present, the conjugates remain bound in the corresponding microwells 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 sample’s coproantigen titre. The enzymatic reaction can be stopped by acidification and the resulting optical density at 450 nm read using a photometer. The signals recorded for the negative control microwells are subtracted from the corresponding positive microwells. A control antigen is provided with the kit so as to validate the test results. This control antigen is composed of lyophilised ground flukes.

### III - COMPOSITION OF THE KIT

- **Microplates**: Two 96-well microplates. Rows A, C, E, and G have been sensitised with specific antibody against Fasciola hepatica and rows B, D, F, and H have been sensitised with the control antibody (polyclonal antibody not specific for the parasite).
- **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 room temperature until disappearance of all crystals. Mix the solution well and remove the necessary volume. Dilute the buffer twentyfold with distilled or demineralised water. Store the diluted solution at 4°C.
- **Dilution buffer**: One 50-ml bottle of 5x concentrated buffer for diluting samples and conjugate. Dilute this concentrated dilution buffer fivefold with distilled or demineralised water. Store the diluted solution at 4°C. If a deposit forms at the bottom of the container filter the solution on Whatman filter paper.
- **Conjugate**: One 500-µl vial of a 50x concentrate of biotin-conjugated anti-Fasciola hepatica antibody. The reagent must be diluted fiftyfold in the dilution buffer.
- **Avidine**: One 500-µl vial of a 50-fold concentrate of peroxidase-coupled avidine.
- **Positive reference**: Two vials containing the reference 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 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.
- **Chromogen solution**: One 2-ml drop-dispenser bottle of the chromogen tetramethylbenzidine. Store at 4°C.
- **Substrate solution**: One 30-ml bottle of the hydrogen peroxide substrate solution. Store this reagent at 4°C.
- **Stopping solution**: One 15-ml bottle of the 1 M phosphoric acid stop solution.

### IV - PRECAUTIONS FOR USE

- This test may be used for *in vitro* diagnosis only. It is strictly for veterinary use.
- The reagents must be kept at between 4 and 8°C. The conjugate must be kept at 4°C. The positive reference must be kept at -20°C once reconstituted. 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.
- Do not use reagents from other kits.

### V - PROCEDURE

1. Bring all the reagents at room temperature at least half an hour before use.
2. Remove the microplate from its packaging.
3. Dilute the concentrated washing solution 20 fold in distilled water. Be sure that all crystals have disappeared before dilution.
4. Dilute the concentrated dilution buffer 5 fold in distilled water. Keep these solutions at 4°C when not used.
5. Dilute the faecal material in the dilution buffer (3 g + 3 ml). Centrifuge 10 min. at 1,000 g.
6. Incubate the plate at room temperature for 1 hour.
7. Rinse the plate with the washing solution, prepared as instructed in Step 3 as follows: Empty the microplate of its contents by flipping it over sharply over a sink. Tap the microplate upside down against a piece of clean absorbent paper to remove all the liquid. Fill all the used wells with the washing solution using a spray bottle or by plunging the plate in an appropriately dimensioned vessel, 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 proceed to the next step.
8. Dilute the necessary amount of the biotin-linked anti-Fasciola hepatica conjugate fiftyfold in the reagent dilution buffer (20 µl of conjugate + 0.98 ml of the reagent dilution buffer per strip).
9. Add 100 µl of the dilute anti-Fasciola hepatica conjugate solution to each well.
10. Cover with cling film and incubate at room temperature for one hour.
11. Remove the cling film very carefully and wash the plate three times with the washing solution.
12. Dilute the avidine-peroxidase conjugate is liquid and must be diluted fiftyfold in the reagent dilution buffer (20 µl of conjugate + 0.98 ml of the reagent dilution buffer per strip).
13. Add 100 µl of the dilute peroxidase-conjugated conjugate solution to each well.
14. Cover with cling film and incubate at room temperature for one hour.
15. Remove the cling film very carefully and wash the plate three times with the washing solution.
16. Prepare 10 ml of indicator solution extemporaneously as follows: Add 12 drops (500 µl) of chromogen to 9.5 ml of the substrate solution (enough for 1 plate). Mix thoroughly, then pipette onto the plate immediately in volumes of 100 µl per microwell. At the time of distributing the chromogen-substrate mixture over the plates the solution must be completely colourless. If a blue colour appears at this stage, this solution must be discarded and a new one made up using clean glassware and equipment.
17. Incubate for 10 minutes at room temperature. This time is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
18. Add 50 µl of stop solution per microwell.
19. 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 cristallize in wells with strong signals and distort the results accordingly.

### VI - INTERPRETING THE RESULTS

- 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.

Tél : 0032(0)84.32.23.77 - Fax : 0032(0)84.31.52.63 E-mail : a.ginter@biox.com

Tél : 0032(0)84.32.23.77 - Fax : 0032(0)84.31.52.63 E-mail : a.ginter@biox.com