IMMUNOLOGICAL DIAGNOSIS OF
FASCIOLOSIS BY ELISA METHOD
IN SERUM AND MILK

240 Reactions
INTRODUCTION

Fasciolosis, or Liver Fluke, is a helminthiasis caused by a trematode, Fasciola hepatica (or F. gigantica, depending on the geographical area), a parasite that settles in the biliary ducts of many different species (ruminants, horses, humans).

The clinical signs of this disease in cattle and sheep are anaemia and enteritis resulting, eventually in cachexia. More often, it progresses slowly and presents as a chronic disease. This disease causes chronic cholangitis (or cholecystitis).

In young cattle (or in lambs), it can be acute or subacute with hemorrhagic phenomena due to the massive migration of the larvae or be toxicoinfectious from pathogens such as Clostridium (necrosing hepatitis) or Corynebacterium (liver abscess), etc...

The economic impact of Fasciolosis is becoming even more important because infestation often develops in a pemicious way; it progresses slowly and is not immediately recognized.

This herd disease can be rapidly diagnosed by serology and easily treated by the appropriate choice of therapy.

For the last 40 years the presence of anti "f2" antibodies in humans has been considered as a serological evidence for the liver fluke (BIGUET and Coll. 1962 and 1965; CAPRON and Coll. 1964). For domestic animals and pets, a hemagglutination method using the "f2" antigen has been revealed more sensitive than the counter-immunoelectrophoresis or the ELSA methods using a non-purified Fasciola extract (SOULE, BOULARD and LEVIEUX 1989). Bibliographical references will be provided upon request.

The test is made reliable by using the "f2" antigen purified from Fasciola extracts. The "f2" antigen is very immunogenic and highly specific for Fasciola hepatica. This kit has been standardised according to the hemagglutination method (HA) developed by the team of Dr LEVIEUX (INRA) as mentioned previously.

This kit allows determining the rate of antibodies directed to Fasciola hepatica. It has been validated for bovine and ovine sera and on bovine milk. Since in most of the cases, the level of antibodies is correlated with the level of infestation, the analysis can be done on individual sera* as well as on pools of 5 or 10 sera or on a pools including the sera of the whole herd. It can be used on tank milk, too.

**This kit enables:**
- to appreciate the sanitary state of the herd concerning the infestation by Fasciola hepatica.
- to estimate the efficiency of an eventual treatment by following the rate of antibodies in time.

* In some rare cases, animals carrying Fasciola hepatica have been observed without developing specific antibodies. A negative individual serology cannot therefore mean an absolute guarantee of the non-infestation of an animal.

PRINCIPLE OF THE TEST

The principle of the test is:
1) The wells of the polystyrene microplate are coated with "f2" antigen (only the even-numbered columns are coated with the specific antigen).
2) The samples to be tested are diluted and incubated in the wells. Any antibodies specific to "f2" antigen present in the serum will form a "f2" antigen antibody immune-complex and remain bound in the wells.
3) After washing, a Peroxidase conjugated anti-ruminant IgG antibody is added to the wells. This conjugate will bind to the immune-complex.
4) After another washing, the enzyme substrate (TMB) is added to the conjugate, forming a blue compound becoming yellow after blocking. The intensity of the colour is a measure of the rate of antibodies present in the sample to be tested.

**Note:** the positive control serum corresponds to a serum that contains ±150 UHA in the unit system of Dr. LEVIEUX (see bibliography).
**KIT CONTENTS and STORAGE OF REAGENTS**

It is recommended to put all the reagents to room temperature (+21°C (± 5°C) at least one hour before use (except the conjugate).

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY</th>
<th>STORAGE AND NOTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biwell Coated microplates</td>
<td>5</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td>- If a microplate is not entirely used, it may be stored for later use, if it is immediately closed in an airtight container and stored at +5°C (± 3°C).</td>
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<tr>
<td>Concentrated wash solution (X 20)</td>
<td>1x 100 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td>- May form crystals at +5°C (± 3°C), but these rapidly disappear at 21°C (± 5°C). A gentle shaking of the solution will accelerate the dissolution of the crystals</td>
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<tr>
<td>- This solution can also be stored at +21°C (± 5°C) for 1 month, if the vials are closed in a watertight way, in order to be immediately available when needed. After dilution, the Wash Solution can be stored for 3 days at +5°C (± 3°C)</td>
<td></td>
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<tr>
<td>- The &quot;Concentrated wash solution (X 20)&quot; is the same for all the kits of INSTITUT POURQUIER and could be used equally in the different kits.</td>
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<tr>
<td>Dilution Buffer 2 light green (for samples)</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
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<tr>
<td>Dilution Buffer 1 light blue (for conjugate)</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
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<tr>
<td>Positive control (liquid)</td>
<td>1 x 1 ml bottle</td>
<td>+5°C (± 3°C)</td>
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<tr>
<td>Negative control (liquid)</td>
<td>1 x 1 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td>Anti-ruminant IgG / peroxidase conjugate</td>
<td>1 x 0,75 ml bottle</td>
<td>+5°C (±3°C)</td>
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<tr>
<td>- The diluted conjugate solution can be stored up to 8 hours after preparation</td>
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<tr>
<td>Revelation solution 3 (TMB) ready to use</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
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<tr>
<td>- This solution can be slightly bluish at +5°C (± 3°C) and becomes colourless at +21°C (± 5°C)</td>
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<tr>
<td>- Thus it can be left on the draining board at +21°C (± 5°C) up to 1 week (if the vial is well closed in an airtight way), in order to be immediately available when needed.</td>
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<tr>
<td>Stop Solution (H₂SO₄ 0,5M solution) ready to use</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td>- It can be stored at +21°C (± 5°C) up to 1 month (if the vial is well closed), in order to be immediately available when needed.</td>
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</tr>
<tr>
<td>- This stop solution is the same for all the kits of INSTITUT POURQUIER and could be used equally in the different kits.</td>
<td></td>
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<tr>
<td>Using instructions</td>
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</table>
PRECAUTIONS FOR USE

1) Do not place the pipette in the mouth when testing reagents.
2) Avoid contact of the substrate (TMB*) with skin, mucous membranes and eyes.
3) «Stop-solution» which contains H$_2$SO$_4$(0,5M) acid, can cause serious burns in case of contact with skin, mucous membranes and eyes.
4) Even if the material delivered in the package does not contain any contaminating element, and that the ruminant sera and milks are, in theory, non-infectious, it is nonetheless advised to decontaminate the whole disposable elements used during the course of handling by immersion for at least of 1 hour in freshly prepared 5% sodium hypochlorite, prior to eliminating them, or to use the autoclave at 120°C for a minimum of 1 hour.

* The Institut Pourquier is at your disposal to supply the toxicity form of the product.

MATERIALS REQUIRED BUT NOT INCLUDED IN THE KIT

1) Microplates reader
2) Centrifuge
3) Centrifuge tubes
4) Vortex
5) Microplate washing system that distributes 300 µl per well
6) Precision Micropipettes and Multi-dispensing micropipettes (The precision required must be lower or equivalent to 5% for all the volumes indicated)
7) Disposable pipette tips
8) Distilled water: the water used for the reconstitution of the controls, the wash solution can come from a conventional distillation system or any other high-performance water purification system (reverse osmosis, resin or activated charcoal purification ...).
9) Microplate covers, aluminium foil or adhesive.
10) Incubator at +37°C (±3°C)

INSTRUCTIONS FOR USE

1) DEPOSITING THE SERA

a) Treatment of controls:
   Controls are diluted to 1/20 by using the following method (see note 2 and 3):
   - Dispense: - 190 µl of "Dilution buffer 2 » per well
   - 10 µl of undiluted negative control in A1 and A2.
   - 10 µl of undiluted positive control in B1, B2 and C1, C2

b) Treatment of serum samples:
   - Sera or pools of sera are diluted to 1/20 by using the following method (see note 2 and 3)
   - Dispense: - 190 µl of "Dilution buffer 2 » per well
   - 10 µl of each serum (individual serum or serum pool) in one coated well (even-numbered columns)
   - 10 µl of each serum (individual serum or serum pool) in one uncoated well (odd-numbered columns)

c) Treatment of milk samples:
   dispense: - 200 µl of each undiluted milk sample in one coated well (even-numbered columns)
   - 200 µl of each undiluted milk sample in one uncoated well (odd-numbered columns)

Note: The positive and negative control samples are diluted to 1/20, including in the case of milk analyses.
d) Homogenize the contents of the wells by gently shaking the plate (see note 1).
e) Cover the plate (with a lid, aluminium or adhesive foil) and leave to incubate for **1 hour** ([± 5 min.]) **at 37°C ([± 3°C])**.

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<td>5</td>
<td>5</td>
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</tbody>
</table>

N = Negative control  
P = Positive control  
1 = Sample n° 1  
2 = Sample n° 2  
3 = .........................

**Figure 1**: Distribution of samples

**Notes**

1) In our laboratories, a microplate shaker originally designed for a complement fixation test micromethod is used.

2) The individual filling of the 96 wells is sometimes a long process. In order to standardize the sample incubation time, controls and samples are prepared in 96 U-shaped-bottom-well plates. It is therefore possible to transfer them rapidly (row by row) by using a multi-channel pipette.

3) The position of the controls in A1/A2, B1/B2 and C1/C2 is not important. They may be dispensed anywhere on the plate.

2) **WASHING**

a) Dilute a bottle of “Concentrated Wash Solution (20x)” in 1900 ml of distilled water. This solution is hereafter called the “Wash solution”. The dilution can be carried out before the elimination of crystals, which appeared at +5°C ([± 3°C]), in condition that the whole 100 ml vial is used.

b) Empty the content of the plate by “flick-off” or better by an automatic method.

c) Fill all the wells of the plate with the wash solution; then empty them again.

d) Repeat the step c) twice (a total of 3 washes).

Note:

1. If milks are skimmed (or taken under the cream), this type of washing is sufficient. With full fat milk, it can be necessary to modify this method of washing: if a visual control reveals whitish traces in the wells, it is advised to leave the solution of washing in contact 1 to 3 times 3 minutes. Indeed, these contact times allow the elimination of fat particles that are likely to fix the conjugate in the next step in a non-specific way.

2. When several plates are processed at the same time, it is possible (in order to synchronize all the steps) to leave the plates full of “Wash solution” during one hour without modifying the validity of the test.

3) **DEPOSITING THE CONJUGATE**

a) Dilute the conjugate to **1/100** with the “Dilution buffer 1” and dispense 100 µl per well.

b) Cover the plate (with a lid, aluminium foil or adhesive) and incubate for **30 min.** ([± 3 min.]) **at 37°C ([± 3°C])**.
4) **WASHING**
   a) Empty the content of the plate by “flick-off” or better by an automatic method.
   b) Fill all the wells on the plate with the wash solution; then empty them again.
   c) Repeat the step b) twice (total of 3 washes)

   **Notes:**
   1) Particular care with the last wash is very important in getting a good test result.
   2) If the washing is carried out by a manual method, it is possible after the last washing to drum the microplate on a dry towel in order to empty the wells completely.

5) **REVELATION**
   a) Dispense 100 µl of “Revelation Solution 3” ready to use per well
   b) Incubate the plate **at 21° (± 5°C) for 20 minutes** (away from the light).
   c) Dispense 100 µl of “Stop Solution” per well.
   d) Shake gently the plate until the coloured solution is homogenized. Wipe carefully the underside of the plate

   **Notes:**
   1. The 20-minute revelation period as outlined above gives the O.D. values provided in the paragraph “INTERPRETATION”, when implemented in our laboratories. Nevertheless the rate of colour revelation can be slightly modified by different factors (quality of the washes, quality of water used, precision of the pipetting, temperature of the reaction...). Depending then on these factors, the revelation step may give O.D. values higher or lower than those expected. In this case, the reaction may be stopped at 20 minutes ± 10 minutes.
   2. The reading can be done up to 1 hour after having stopped the reaction on condition that the plates are kept in the dark.

6) **READING**
   a) Read the optical densities at 450 nm (OD.450). The photometer must first be blanked on air.
   b) Calculate the **corrected OD.450** for each serum sample: Subtract the OD 450 value obtained from an uncoated well from the OD.450 from a coated well.

**VALIDATION CRITERIA**

The results can be considered reliable if:

- The mean uncorrected OD 450 of the positive control is > **0.350**

  and

- A ratio between the mean corrected OD 450 value of the positive control and corrected OD 450 value of the negative control is greater than or equal to **3.50**.

**Notes**

1) The corrected OD 450 value of the negative reference can be found “negative” or equal to zero. In this case, used the absolute value for the validation.

2) If the corrected OD 450 of the negative reference is 0, to calculate the ratio use the value of 0.001
INTERPRETATION

Calculate for each sample, the S/P ratio:

$$\%S/P = 100 \times \frac{\text{corrected OD450 value of the sample}}{\text{mean corrected OD450 value of the positive control}}$$

<table>
<thead>
<tr>
<th>S/P % of the sample</th>
<th>Correlation between test result and infestation level (individual sera)</th>
<th>Correlation between test results and the proportion of infestation within the herd (pools of sera or tank milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$%S/P \geq 150%$</td>
<td>+++</td>
<td>Strong Infestation ($&gt;50%$ of infestation)</td>
</tr>
<tr>
<td>$80 &lt; %S/P &lt; 150%$</td>
<td>++</td>
<td>Medium Infestation ($20%-50%$ of infestation)</td>
</tr>
<tr>
<td>$30 &lt; %S/P \leq 80%$</td>
<td>+</td>
<td>Low Infestation ($&lt;20%$ of infestation)</td>
</tr>
<tr>
<td>$%S/P \leq 30%$</td>
<td>0</td>
<td>No or very weak infestation</td>
</tr>
</tbody>
</table>

Notes:
1. The values described in the above table are only indicative. Indeed, in case of analysis of serum or milk pools, the results depend on the antibodies concentration of each sample included in the pool.
2. A positive serology after the return in cowshed reveals the presence of infected animals and pasture land. Even correctly treated with an efficient product, the animal will remain carrier of antibodies during about 12 weeks (this delay changes more or less with the initial rate of antibodies).
3. An animal treated in autumn (in the return in cowshed) cannot be differentiated from an untreated animal before about 12 weeks. On the other hand, a late serology (at the beginning of the spring) will enable to estimate the efficiency of a treatment.

BIBLIOGRAPHY