

# finalreport

Animal Health and Welfare

**Project code:** AHW.021  
**Prepared by:** Dr Gareth W Hutchinson  
Elizabeth Macarthur  
Agricultural Institute  
NSW Agriculture  
**Date published:** July 2003  
**ISBN:** 0 7347 15102

**PUBLISHED BY**  
Meat & Livestock Australia  
Locked Bag 991  
NORTH SYDNEY NSW 2059

## Validation of French Antibody ELISA for Liver Fluke

### Abstract

Existing faecal egg tests for liver fluke (*Fasciola hepatica*) in cattle are inaccurate, while a blood test (ELISA) to detect antibody used in NSW Agriculture laboratories lacks sensitivity. This project validated the Institut Pourquier (France) antibody-ELISA kit under Australian conditions. Over 1 500 samples were tested from artificial and natural infections, including paramphistomes (conical flukes) and tropical liver fluke (*F.gigantica*).

The test had high specificity (>95%) and sensitivity (99%). It was substantially more sensitive than the existing blood test. Paramphistomes did not interfere with its accuracy, nor did previous nematode (roundworm) infection affect responses to later fluke infection. An established in-house test and the commercial kit detected antibodies in early immature liver fluke infection, but kit positives were earlier (2 weeks), and reached maximum levels sooner (8 weeks). Positive results continued for 18 months without reinfection. There was no spike in antibodies when flukes matured, and no relationship between amount of antibody and numbers of adults or eggs.

Pourquier kit is easy to use and is suitable for veterinary laboratories with an ELISA microplate reader. There is good standardisation and quality control of reagents. Kit can also detect antibodies in milk (requires further validation for Australia).

Fluke infective stages (metacercaria) are eaten by cattle mainly from October to May. So, when used in fluke control programs to test cattle herds (beef or dairy) or individual animals, newly infected stock will not react strongly until after late November.

Limitations are typical of antibody-ELISAs: inability to distinguish between current and previous infections, and potential for cross-reactions. Until new generation tests (antigen-detection or molecular procedures) are widely available, it will be useful.

### Executive Summary

Liver fluke, *Fasciola hepatica*, is a snail transmitted parasite of sheep and cattle in Australia which has been estimated to result in 5% average loss of production and some \$20 million annual costs of chemical treatments. Current parasitological tests to detect liver fluke by finding parasite eggs in faeces are insensitive, time consuming and can only detect infections some 12-14 weeks after infection.

An antibody-based test (ELISA) has been developed by a French research company Institut Pourquier, and is now available commercially in Australia. The test is claimed to be highly specific and sensitive in detecting liver fluke infection in cattle. However, limited validation of the test under Australian conditions has been conducted.

This project was designed to further assess the usefulness of the Pourquier test for use in Australian cattle.

The aims of the project were to use the Institut Pourquier ELISA kit to determine:

- The sensitivity and specificity of the test in a range of experimental and natural infections;
- Whether the test can be used to distinguish between infections with liver fluke and stomach flukes (paramphistomes);
- The concentration of antibodies over time following the termination of liver fluke infections by chemotherapy;
- The degree of cross-reactions in the test with other parasite infections in cattle;
- The time course of antibody responses following infection, and the sero-conversion in prepatent infections;
- And if possible to determine the relationship between antibody levels and fluke burdens or faecal egg counts.

The effectiveness of the kit was compared with an existing in-house antibody ELISA used by NSW Agriculture.

A series of studies was conducted using available serum banks from experimental and natural infections of cattle held by NSW Agriculture. Additional batches of sera were obtained from cattle believed to be free of liver fluke from a non-endemic region of Western Australia, and from cattle infected with a related parasite, *F. gigantica* from Indonesia. Over 1500 individual sera were tested.

The Institut Pourquier ELISA kit for liver fluke in cattle has a high specificity (95.3 to 98% depending on samples) and sensitivity (99%). It appears to be substantially more sensitive than the NSW Agriculture ELISA (60% sensitivity). Using the manufacturer's recommended positive cut-off of 30% Sample/Positive control ratio gives adequate sensitivity.

## Contents

	Page
<b>1</b>	<b>Background..... 5</b>
<b>2</b>	<b>Objectives..... 5</b>
<b>3</b>	<b>General Methods..... 6</b>
3.1	Pourquier Kit and NSW Agriculture Methods .....6
3.2	Serum Banks .....6
<b>4</b>	<b>Results..... 6</b>
4.1	<b>Objective 1: Determine Sensitivity and Specificity .....6</b>
4.1.1	Specificity - True negatives .....6
4.1.2	Sensitivity: .....7
4.2	<b>Distinguishing Between Fasciola and Paramphistome Infections 7</b>
<b>5</b>	<b>Benefits to the Meat Industry ..... 7</b>
<b>6</b>	<b>Discussion..... 8</b>
<b>7</b>	<b>Conclusions and Recommendations - Section 8</b>
<b>8</b>	<b>Bibliography ..... 10</b>
<b>9</b>	<b>Appendices..... 10</b>
9.1	Appendix 1 .....10

# 1 Background

Classical parasitological faecal examinations of cattle for the presence of liver fluke *Fasciola hepatica* eggs, are renowned to be limited in usefulness as they can only detect patent (egg producing) infections with adult flukes. There is a long pre-patent period of some 12-16 weeks after infection before eggs are detectable, and the tests are inaccurate (for example the standard Happich & Boray sedimentation test only detects approximately 30% of infections) due to low levels of eggs produced and non-consistent shedding of fluke eggs.

To improve the diagnosis of fasciolosis in cattle many serological studies have involved use of antibody detection systems to *F.hepatica* infections. Among these is the currently available Ab-ELISA in use in the NSW Agriculture laboratory at EMAI originally developed by Rolfe and coworkers, which uses a partially purified adult fluke somatic antigen. During the early 1990s studies by Pourquier et al. (1995) in France developed an antibody detection ELISA for *Fasciola hepatica* based on the use of the F2 excretory/secretory antigen for capture of antibodies, and this test has been commercialised as a kit, now available in Australia. The manufacturers claim good specificity and sensitivity for serum detection in cattle.

Studies recently completed in Australia (Reichel, 2002) have reported that the Pourquier test has a specificity of 100% and a sensitivity of 100 % in cattle. However, this study did not address the major problem of cross-reactions caused by animals infected with stomach fluke, which is a major parasite either separately or concurrently with liver fluke infecting animals particularly in coastal parts of NSW, and regional parts of Victoria. Nor did it evaluate the declining antibody levels in cattle following anthelmintic treatment and there was no correlation with adult fluke burdens or faecal worm eggs per gram.

# 2 Objectives

Objective of this preliminary project is therefore to resolve the question of whether the Institut Pourquier test is suitable for use in Australia to detect *Fasciola hepatica* infections in cattle. In particular, to determine:

- Its sensitivity and specificity in a range of experimental and natural infections.
- If it can be used to distinguish between infections with liver fluke and paramphistomes
- Time course of antibodies following termination of natural or experimental liver fluke infections by chemotherapy (antibody persistence)
- Cross reactions with other parasite infections in cattle
- Earliest detection of seroconversion by infected animals, and if possible the relationships between antibody levels and fluke burdens/faecal egg counts

Should the project be substantially successful in its objectives the intention would be for this test to become the de facto standard method for liver fluke detection in cattle in Australia, and its use be endorsed by MLA. Improved and cost effective diagnosis would ensure more accurate assessment

of the importance of liver fluke in cattle production and timely and effective treatment leading to reduced costs of production to producers.

### 3 General Methods

#### 3.1 Pourquier Kit and NSW Agriculture Methods

---

All serum samples were tested at the EMAI laboratory according to the package inserts of the Institut Pourquier kit without modification (see Attachment). This to determine specificity and sensitivity, the absorbance values of samples were compared to that of low positive control sample (equivalent to 150 HA units) provided with the kit, and plotted as Sample to Positive ratios (S/P%). Values greater than 30% of the provided kit positive control samples are considered positive according to the kit methodology. Results were also compared with the more usual ELISA units or optical densities using the in-house NSW Agriculture ELISA. Values of >40 EU, are considered positive in this assay, since it's re-validation in 1999. At this cut-off the sensitivity is 60% and the specificity is 97.5%

The same serum samples were tested using the NSW Agriculture *F.hepatica* antibody ELISA, using as standards high fluke positive sera and know worm (nematode) infected and paramphistome infected sera as negative controls. The values were determined in ELISA units according to NATA-authorized standard operating procedure (MPAR-010.2)

#### 3.2 Serum Banks

---

Cattle serum used in this study have been collected from animals infected both naturally and experimentally, as well as animals raised fluke-free, and in some cases nematode free. Sera have been stored either at -20 or -80°C for periods up to 19 years without preservatives. The following serum banks/experimental and field infections were studied.

### 4 Results

#### 4.1 Objective 1: Determine Sensitivity and Specificity

---

A number of different sources were used for this component.

##### 4.1.1 Specificity - True negatives

192 sera were provided by Dr Dieter Palmer, Department of Agriculture WA, from animals from non-endemic area of southwestern WA.

Sera from 8 cattle on 24 different properties were tested using the Pourquier kit. A total of 9 samples gave reading greater than the 30% Sample/Positive ratio manufacturer's recommended cut-off (mean  $51 \pm 25$  (SD)), with the average of 183 negative samples having a reading of  $-1 \pm 13$ . Specificity was determined as  $183/192 = 95.3\%$  at this cut off value.

### 4.1.2 Sensitivity:

Sera from NSW Agriculture Milk ELISA validation trials (1994-96), includes 285 sera from 3 farms with endemic liver fluke infections. Fifty-three of these sera samples had paired faecal samples. Sixteen were confirmed to have liver fluke eggs in faeces, and 50 had eggs identified as paramphistomes, 15 had both liver fluke and paramphistome eggs and three had neither.

Only seven animals from one farm had kit ELISA results with less than 30% S/P ratio, with an average of  $8 \pm 9$  S/P%. Five of these had faeces tested, two had no detectable eggs of either type, while three had only paramphistomes. When tested using the in-house ELISA in 1996 when the positive cut off was  $>45$  EU, all five gave ELISA values of 17-36 EU. It would therefore appear that these are true negatives for liver fluke based on the criteria of both ELISA tests and the faecal egg sedimentation.

## 4.2 Distinguishing Between Fasciola and Paramphistome Infections

---

Sera from a cattle paramphistome epidemiology study (PF Rolfe 1991) on North Coast, NSW were used. An additional 10 sera from dairy cattle on the south Coast of NSW were obtained in collaboration with Dr A Carr, DV South Coast, Bega RLPB, with confirmed paramphistome infections based on faecal egg counts, but negative for *Fasciola*.

The paramphistome epidemiology sera included samples from groups of 10 individual tracer animals grazed for 14 different 8 week periods from Oct 1983 to Nov 1985 on a property with confirmed paramphistome infections, known to be *Fasciola*-free on the basis of faecal egg counts.

## 5 Benefits to the Meat Industry

The Pourquier ELISA kit is easy to use and is suitable for diagnostic parasitology laboratories equipped with an ELISA microplate reader. It is suitable for use in Australia, and would be a good additional test to be offered by both government and private veterinary laboratories, which currently do not have access to serological tests for liver fluke.

The cost of testing is marginally more expensive than existing in-house ELISA offered as a commercial service by NSW Agriculture. Good standardisation and quality control of reagents makes it an attractive alternative test, particularly as it can also be used to detect liver fluke antibodies in milk (provided adequate further validation is conducted).

Test is suitable for use for group testing of cattle herds (beef or dairy) or individual animal testing due to high specificity and sensitivity. With high probability of individual infection in an exposed herd, and with expected prevalence of 30–50%, based on herd size of 200 head, 21 to 13 animals should be tested for statistical reliability.

Test should be an integral part of a control program for liver fluke. Seasonality of infective stages (metacercaria) on pasture is predominantly from October to May, Fluke-naïve animals would not be expected to become strongly sero-reactive in the Pourquier ELISA until late November onwards.

Limitations of the Pourquier test are characteristic of all IgG antibody ELISAs: primarily the inability to differentiate between active and previous infections, and potential for cross-reactions with other parasites.

## 6 Discussion

The results of this study confirm that the antibody ELISA is a sensitive and specific method of detecting *Fasciola* infections in cattle. Differences in the results obtained by the two methods may be attributed in part to the differences in the antigens used for the tests; being a purified f2 antigen in the case of the Pourquier kit and a fractionated somatic antigen in the NSW Agriculture in-house method. There are also differences in the source of the conjugate, being anti-bovine IgG in the in-house assay and a more non-specific anti-ruminant IgG in the kit. Neither assay used a sub-class specific IgG1 or IgG2 conjugate. Attempts by Palmer (unpublished) to modify the in-house test by using only IgG1 specific antibodies improved the specificity but there was some loss of sensitivity. The positive reference sera used in the two methods are also different. A highly reactive positive control serum is used in the in-house method and a low positive control (= 150 HA units) in the kit, so that results where EU = 100 are not equivalent to 100% S/P in the kit. This difference is important in considering the time at which sero-conversion may take place following infection.

The levels of specificity and sensitivity reported here with the Pourquier ELISA are slightly lower than those reported by Reichel (2002) using the kit. However, he used a much higher positive cut-off of 77%S/P (parametric method), when the version of the kit he used (in 2001) had a manufacturer's recommended cut-off value of 100%. Since that time the manufacturer has modified the kit to make it useable for both serum and milk and the recommended cut-off has been reduced to 30%S/P. This was the value used in the present study.

## 7 Conclusions and Recommendations - Section

- The Institut Pourquier ELISA kit for liver fluke in cattle has a high specificity and sensitivity. It appears to be substantially more sensitive than the NSW Agriculture ELISA. Using the manufacturer's recommended positive cut-off gives adequate sensitivity.
- Presence of paramphistome infection does not appear to significantly interfere with the accuracy of the test, but further studies with experimental stomach fluke infections in parasite-naïve calves would be required to confirm this.
- The kit detects seroconversion within a few weeks of infection. The length of persistence of antibody following **effective** treatment (in the absence of natural re-infection) requires better-controlled studies to determine the exact length of period, provided it is possible to ensure that flukes are killed by the treatment.
- Previous nematode infection did not result in false positive results, nor did such infections, when chemically abbreviated; restrict the ability of the ELISA kit to detect subsequent liver fluke infection. Concurrent nematode infections in natural fluke infections were not examined specifically. Incidental findings are that they do not cause cross-reactions.

SAMPLE ONLY

## 8 Bibliography

Anderson N, Luong TT, Vo NG, Bui KL, Smooker PM and Spithill TW (1999). The sensitivity and specificity of two methods for detecting *Fasciola* infections in cattle. *Veterinary Parasitology* **83**: 15-24.

Bossaert K, Farnir F, Leclipteux T, Protz M, Lonneux J-F, and Losson B (2000). Humoral immune response in calves to single-dose, trickle and challenge infections with *Fasciola hepatica*. *Veterinary Parasitology* **87**: 103-123.

Castro E, Freyre A and Hernandez Z (2000). Serological responses of cattle after treatment and during natural re-infection with *Fasciola hepatica*, as measured with a dot-ELISA system. *Veterinary Parasitology* **90**: 201-208.

Greiner M, Sohr D and Gobel P (1995) A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. *Journal of Immunological Methods* **185**: 123-132.

Hillyer GV (1999). Immunodiagnosis of human and animal fasciolosis. In **Fasciolosis** (editor JP Dalton), pp 435-447, CAB International, Wallingford, UK.

## 9 Appendices

### 9.1 Appendix 1

Figure 1. LPC Negative Property

