

final report

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Validation of French Antibody ELISA for Liver Fluke

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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

Background

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. By this time considerable damage to the liver has already been caused. Approximately 20,000 tests are performed annually in Australia for diagnosis of infection, monitoring of control programs and certification for movement of livestock to fluke-free areas (Western Australia).

Serological (blood) tests have been developed which are capable of detecting antibodies produced in response to infection. These tests are useful as herd tests but not for individual animals. They are good at confirming freedom from disease (high specificity) but less reliable in accurately assessing infection (relatively low sensitivity). Antibodies persist for months after fluke have been killed or died naturally.

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.

Objectives

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.

Activities

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. Some studies involved experimental infection of fluke-free calves maintained parasite free and away from possible reinfection for periods up to 18 months. The effects of chemotherapy on antibody levels were observed in some animals, and others were from epidemiological projects involving tracer calves exposed to natural

infections of paramphistomes. Numerous sera from dairy cattle naturally infected with liver fluke from coastal areas of Southern NSW were also investigated.

The sera were tested with the Pourquier ELISA according to the manufacturer's instructions and the results compared with values produced by the NSW Agriculture in-house ELISA. This latter test uses standards of high fluke-positive sera and known paramphistome infected sera and nematode (worm) infected as negative controls. In some instances, historical data were also available on the infection status of the cattle as determined by fluke faecal egg counts or adult fluke counts.

Major Outcomes

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.

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 (2–4) 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.

Limited studies with serum from animals infected with the related tropical liver fluke *Fasciola gigantica* indicate that the Pourquier ELISA kit may be used to detect this parasite in overseas countries.

The pattern of serological responses in cattle experimentally infected with *F.hepatica* was similar whether using the Pourquier Kit or the NSW Agriculture ELISA. IgG antibodies were detected earlier with the kit (2 weeks after infection), and reached maximum levels sooner (by 8 weeks). Persistence of positive titres continued to at least 18 months in the absence of reinfection.

There was no apparent spike or increase in antibodies detected by the kit that could be attributed to the maturity of adult liver fluke at the time of patency (production of eggs).

There was no relationship between positive kit results and either numbers of adult flukes or counts of fluke eggs in faeces.

Recommendations and 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.
- The kit is not suitable for crush/race-side detection in its present form. Considerable development and conversion of the method to a immunochromato-graphic system would be required to facilitate crush-side diagnosis. The advantages of immediate diagnosis in expediting treatment in cattle are not obvious due to withholding periods and residue issues limiting available drench options particularly in lactating cows.
- The test is not suitable for barrier exclusion purposes despite the relatively high specificity as there is no correlation with shedding of fluke eggs.
- There are still grounds to support the development of alternate diagnostic tests for liver fluke, such as antigen-detection methods or molecular procedures. Such tests have been attempted using purified natural and synthesised antigens overseas, and although claims are made for their reliability and ability to accurately determine success of chemotherapy, they are not readily or widely available.

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1. COMMUNICATIONS STRATEGY

The primary targets for the outcomes of this study will be to inform veterinary diagnostic laboratories of the advantages of this test for the diagnosis of liver fluke. Key influencers will be the OICs of the regional and central veterinary laboratories in all states.

The executive summary has already been forwarded to the NSW Agriculture Program Leader for Diagnostic and Analytical Services, and the three OICs of the Regional Veterinary Laboratories in Orange, Menangle and Wollongbar, with a recommendation that the DAS Program adopt this test as a replacement for the existing in-house ELISA.

I have been informally advised that at least the central Diagnostic Parasitology laboratory at EMAI will be switching to this test in the near future and they are currently evaluating the test for milk.

Once the final report has been approved by MLA it is proposed to:

- Advise the agency which distributes the kit in Australia (Laboratory Diagnostics Pty Ltd) of the findings and provide them a copy of the full report. (October 2003)
- Write a short Agfact on **Improved Liver fluke Diagnosis using the Pourquier Antibody ELISA**. This publication will be published on the NSW Agriculture web site and promulgated via the electronic **WormMail** newsgroup mediated by the NSW Agriculture State Worm Control Coordinator. This electronic newsgroup is distributed to District Veterinarians, Private vets, the major Agricultural and Veterinary Chemical Companies, CSIRO, and major rural stock and station agencies (such as Wesfarmers Landmark, Elders, CRT and IAMA).
- An article for **Agriculture Today** (an insert in **The Land**) by November 2003;
- An article in the next edition of **Turning the Worm** Newsletter published by NSW Agriculture (January 2004).
- As part of a review of the **Australian Standard Diagnostic Tests for Animal Diseases** (ASDT), published by the Sub-Committee on Animal Health Laboratory Standards (SCAHLs), a separate section on Liver Fluke Diagnosis is being written by Dr G Hutchinson, and the IP liver fluke ELISA will be described and its key features highlighted (early 2004).
- The report will also be distributed to the Chief Laboratory/Veterinary Pathologists in Departments of Primary Industries/ Agriculture the Victoria, Queensland, Tasmania, South Australia and Western Australia. Copies of the executive summary and links to the MLA website will be sent to the major private veterinary diagnostic laboratories (IDEXX-VPS, ParaSite Diagnostic Services, VHR and Gribbles Veterinary Pathology Labs).
- A scientific paper is being prepared for publication in the journal **Veterinary Parasitology**, with a target date for submission of December 2003.
- A conference presentation is planned for the next scientific meeting of the Australian Society for Parasitology to be held in Fremantle, WA in 2004. A small summary on the test will be submitted to the Newsletter of the Society, to be published in November 2003.

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3. 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.

4. 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.

5. GENERAL METHODS

5.1. 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)

5.2. 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.

6. RESULTS

Objective 1: Determine Sensitivity and Specificity.

A number of different sources were used for this component.

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 ± 25 (SD)), with the average of 183 negative samples having a reading of -1 ± 13 . Specificity was determined as $183/192 = 95.3\%$ at this cut off value.

Forty-seven sera from confirmed fluke-negative dairy farms in the Camden/Leppington area were also tested, and compared to results with the same samples using the in-house test in 1995. The results are shown in Figure 1. One sample was considered positive in the kit (36% S/P), and one recorded marginally above the cut-off of >45 EU set at the time. The animals recording positive results were not the same. The specificity of both tests at the given cut-offs, assuming the positives to be false positives is 98%.

These results confirm the capacity of the antibody ELISA, whether the in-house test or the Pourquier kit to reliably confirm the absence of infection, in the great majority of cases. The levels report in the present study, confirms earlier findings with the in-house test (Rolfe, Young and Loughlin 1993), but are slightly less than the 100% claimed by Reichel (2002) for the Pourquier kit, when he selected 76.7% S/P as an appropriate cut-off using a parametric method, and 54.3% using a non-parametric method in the two graph receiver operating characteristic (TG-ROC). At that time the manufacturer's suggested cut-off was 100% S/P. In the current version of the kit (PO5120/01-21/11.02), which is now modified for both serum and milk, the recommended cut-off is 30%. This was the value used in the present study.

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 ± 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.

The 278 animals with positive kit responses were strongly positive with results usually well in excess of the kit low HA positive control (100% S/P) and had average values of 137 ± 22.5 S/P%. Assuming that five of the seven negative results are true negative and the other two are false negatives, then the sensitivity of the kit was determined as $278/278 + 2 = 99.3\%$

The S/P% values for the 53 animals which had faeces examined are shown in Figure 2, and compared to the EU values of the samples when collected in 1995. Sixteen of the 53 samples had values less than the positive cut-off as determined by the in-house ELISA, indicating that the sensitivity, at least in these farms, was less than that of the kit, and less than the 95.7% previously determined when there was an estimated prevalence of 3.8% false positives (Rolfe et al 1993).

From the South Coast region of NSW, another three farms with a total of 63 sera with matching faecal

samples were further studied. These had been adjudged infected with liver fluke based on the in-house ELISA in 1994, with the exception of one animal. Farm A had 20 out of 20 positive using the Pourquier kit, mean 138 ± 23 S/P%; Farm B 29/29 positive with a mean of 124 ± 23 S/P%; and Farm C with 14/14 positive with mean of 113 ± 29 S/P%. The one animal previously believed to be negative as it had no detectable fluke or paramphistome eggs and negative serum with 29 EU in 1994, was positive with the kit at 62% S/P.

Farm A had all 20 with paramphistomes eggs and 18/20 with *Fasciola*, Farm B 17/29 with paramphistomes and 25/29 with liver fluke, and Farm C 14/14 with stomach fluke and 11/14 with *Fasciola* eggs.

When only those with confirmed *Fasciola* FEC-positive status were compared, there was no significant difference in the kit ELISA results from the overall means.

The difficulties of interpreting true liver fluke infection status on the basis of ELISA results in field or natural infections, when mature animals (especially dairy cows) are grazing pastures suitable for both liver fluke and stomach fluke snails, is apparent from the above. As most farmers in endemic regions will have some treatment programs for liver fluke, the finding of paramphistome eggs in faeces in the absence of *Fasciola*, does not imply that elevated serum ELISA values are due in part to the stomach fluke infections. Interpretation of ELISA results using the Pourquier kit are probably more reliable than the in-house test in concurrent infections. The problem of interpretation of paramphistome infections is discussed further in the next section.

Objective 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.

Sera from the following animals at pre and post 8 weeks grazing. The numbers are believed to correspond with the grazing periods. Necropsy data and average paramphistome counts, indicate that periods 2 and 3 (Dec 1983-March 1984) and 10-13 (March 1985-Sept 1985) had highest paramphistome burdens ranging from 4,000 to approx 20,000 per animals.

Paired pre-post infection sera numbers 21-30, 31-40, 41-46, 51-59, 61-69, 81-90, 91-96, and 101-109 (some missing individuals). A total of 130 sera were tested.

Matching of the animal numbers on sample tubes with the data on grazing period given in Table 3.1 and 3.2 (Rolfe 1991), has proven problematic. It is assumed that they correspond with periods 2 through 10.

The mean ELISA values using the kit and those reported on the same samples by Dr Palmer, using the NSW Ag in-house fluke ELISA, are given in Table 1, and shown graphically in Figure 3.

At the time before the animals were placed on pasture (labelled "prebleed") of animals 21-29, six animals were detected as positive to *Fasciola* using the kit, and four in the in-house assay. After eight weeks six were positive in both assays and one by the kit alone. Animals 30-39 were negative at prebleed by both methods and one became positive at 8 weeks by the kit. Animals 40-46 had four positives at prebleed and four by both methods at 8 weeks and one using the kit alone. Animals 51-59 had only one animal positive by the kit at prebleed and none at eight weeks.

Groups 81-89 and 90-96 had three positive at prebleed using the kit, but at eight weeks these were negative, except for one animal which was positive by the in-house ELISA.

All weaners in groups 61-69 and 101-109 were negative at both periods by both methods. Overall six animals sero-converted in the 8 weeks of grazing as tracers (five by kit and one by in-house).

The finding of a substantial proportion of animals in the group 21-29 having high antibody levels when first placed on the trial pasture, suggests that they must have been exposed to liver fluke at an early age. (If the animal numbers correspond to Grazing period 2 (Nov-Jan 1983-84). According to the records when slaughtered after 8 weeks this group had a very high paramphistome burden with a geometric mean of 193,222 (Rolfe 1991). If however, the sample labelling is incorrect, it is possible that these animals are actually from grazing period 1, and these were 9-12 month old (Table 3.1 in Rolfe 1991). Although all animals were claimed to be fluke-free based on faecal egg counts when put out on the trial pasture, the possibility arises of them being in the prepatent phase of an infection acquired earlier than the study. Although all groups had paramphistome infections when slaughtered after the eight-week tracer period, there is no mention in the report of the trial of any liver fluke being detected.

Additional sera from South Coast NSW properties with confirmed paramphistome infection on the basis of faecal egg counts are shown below in Table 2.

Table 2: Liver fluke ELISA results using in-house and commercial kit in animals with confirmed stomach fluke infections.

Accession	Sample	Age	Liver Fluke EPG	Stomach fluke EPG	FLUKE ELISA	
					In-House EU	Kit S/P%
CP2028/1	34C	5 yo	0	98	28	128
2	36H	1 yo	0	12	10	9
3	26	1yo	0	19	11	15
4	0026C	3 yo	0	51	18	-3
5	32H	1 yo	0	30	21	1
6	50C	4.5 yo	0	195	14	14
7	36C	5 yo	0	5	25	132
8	42	2 yo	0	3	15	78
CP1412/1	1	4 yo	0	3	6	7
2	2	4 yo	0	133	10	-1

All animals were negative according to the in-house ELISA, with values substantially less than the current 40 EU cut-off. Older cows did have slightly higher values. Paramphistome egg counts appeared unrelated to ELISA values with either the kit or in-house methods. Two of the ten animals had positive results with the commercial kit, with values well over 100% S/P. As both of these were mature (5 yo) cows, it is likely that they have had *Fasciola* infections at some time in the past near term, which have either been treated or not become patent. All the young heifers and one cow, along with two adult cows from a second property had very low negative results, despite having moderate to very high stomach fluke egg counts.

This supports the belief that stomach fluke infections per se do not interfere with the ELISA kit. However, absolute proof of this would need artificial experimental infections with paramphistome metacercariae in calves raised both worm and fluke-free from birth.

Objective 3: Time Course of Antibody Persistence Following Anthelmintic Treatment.

This study used sera from an experimental study (PR10) in which groups of adult dairy cattle were experimentally infected with *Fasciola hepatica* metacercaria and treated with anthelmintic (Details provided by Dr Rolfe). Trickle infections of a total of 1800 metacercaria were given over a period of 4-6 weeks to adult dairy cows. A total of 22 bleeds were conducted over a period from April 1990 to August 1991 and sera are available from 12 infected and 13 non-infected control animals at these times.

Cows were treated with triclabendazole at week 40 post-infection. They have also been evaluated by Dr D Palmer, Dept of Agriculture, Western Australia with the in-house ELISA. Data on faecal egg counts was retrieved from laboratory worksheets in the NSW Agriculture archives to match fluke infection levels with antibody titres. Some of these results also meet Objective 5 (Time course study).

Approximately 160 infected and 160 uninfected sera were available.

The results of the testing of the uninfected control cows using both the Pourquier kit and the in-house ELISA over the 58 weeks period of the trial are shown in Figure 4. A total of 163 samples were tested using the kit with a mean S/P% of 10 ± 15.3 . Generally, means remained at low and negative values, by both tests, with similar patterns over time.

Cow number 259 was above the cut off of 30% on two occasions (42 and 31 SP%), #404 on four tests (46, 51, 37 and 46 SP%), #501 seven times (36, 33, 42, 43, 46, 50 and 49cS/P%) and #955 five times (55, 49, 50, 47 and 41). Faecal egg counts apparently of one egg were detected on two separate occasions. As the cows were mature age, it is possible that there was a very low background level of *Fasciola* infection in the herd, although it was thought to be fluke-free.

Results of the twelve artificially infected cows are shown in Figure 5. Mean antibody levels were quick to rise to positive cut off values between 2 to 4 weeks post-infection with both the kit and the in-house test. They reached peak values and plateaued at 8 weeks using the kit and slightly later at 12 weeks using the in-house ELISA. Levels remained at this level at least until week 46 (some 6 weeks after treatment) but had declined slightly from the peak by week 58 (3 months post-treatment).

Faecal egg counts were recorded as early as 8 weeks after first infections in 4 of the 12 animals (lower panel Figure 5), which corresponded to near peak antibody levels. Faecal egg counts fluctuated within a range of 1 to 74 epg in individual animals, with no consistent pattern of shedding. However, the average epg rose from 1.5 at 8 weeks to 30.4 at week 20, with all twelve cows shedding fluke eggs from week 32. Despite treatment at week 40 with triclabendazole, liver fluke eggs were still being shed in 10/12 animals at week 50 although average counts were low, and even 18 weeks post treatment 3/12 were shedding. It is clear that the treatment with triclabendazole against this isolate was only partly successful, possibly due to the chronic nature of the infection. This was revealed when the original data sheets from 1991 were examined and it was found that when four cattle were necropsied they had extensive calcification and 100-220 adult flukes, 22 weeks after the treatment.

The persistence of antibodies after treatment using the kit method supports, to some extent, the claim (Rolfe et al 1993) that antibody levels may persist for upwards of 20 weeks. However, better-controlled experiments with fully effective treatment would be needed to be precise about the time period. A major problem in determining this from field cases would be that treated animals are not usually prevented from re-infection, and that as antibody levels increase rapidly within a few weeks of infection, it is difficult to distinguish between persistent antibodies from newly produced antibodies during the prepatent phase.

Objective 4: Cross-Reactions with Other Parasite Infections

Nematode infections

A group of five calves raised worm-free and housed were infected with pure cultures of nematodes (*Ostertagia ostertagi*, *Cooperia oncophora* and *Trichostrongylus axei*). After some 5-6 months, these nematode infections were terminated by benzimidazole anthelmintic treatment, and they were then infected with 250 metacercaria of *F.hepatica*. Blood samples were collected from these animals at weeks 0, 4, 5, 6 and 9 post-infection and tested with the Pourquier kit. The results are shown in Table 3.

Table 3. Effect of previous infection with nematodes on liver fluke ELISA following superimposed infection with 250 *F.hepatica* metacercaria.

Days	Cattle	Kit	Mean
PI	no	S/P%	± SD
0	2	0	-11.2 ± 22.4
	4	2	
	5	-51	
	9	-6	
	10	-1	
30 days	2	118	117.5
	10	117	
35 days	2	133	117 ± 10.3
	4	107	
	5	110	
	9	116	
	10	121	
42 days	2	129	115.2 ± 11.1
	4	109	
	5	103	
	9	110	
	10	125	
65 days	2	124	116.2 ± 7.0
	4	113	
	5	108	
	9	113	
	10	123	

Previous nematode infection did not produce positive responses in the kit ELISA, nor did it affect the antibody response of the cattle to subsequent *Fasciola* infection. Two of the weaner calves had sero-converted when tested on day 30 after infection and all five had strong responses on day 35. Antibody levels persisted at high levels until the trial ended after 9 weeks.

This study did not test the effects of concurrent nematode infection on the ability of the Pourquier test to identify liver fluke infected cattle. However, indirect evidence from the paramphistome epidemiology study (Objective 2), where there were high nematode counts in some of the cattle in tracer groups but these did not appear to influence fluke antibody levels, supports the belief that cross-reactions between nematodes

and liver fluke do not compromise the test.

***Fasciola gigantica* infections**

Fasciola gigantica is a closely related liver fluke that replaces *F.hepatica* in much of the tropical and sub-tropical regions of the world. It is a particularly important pathogen in cattle and buffalo in SE Asia. A small number of serum samples were kindly provided by Dr David Piedrafita (University of Melbourne), from cattle examined in abattoirs in West Java, Indonesia. A number of merino sheep artificially infected with *F. gigantica* were also tested. The results are given in Table 4.

Table 4. ELISA kit values in cattle (natural infection) and sheep (artificial infection) from Indonesia with *Fasciola gigantica*.

Group	Animal ID	Number of adult <i>F.gigantica</i>	Kit ELISA S/P %	Mean \pm SD
Cattle				
1	1	0	15	69.3 \pm 66.1
	2	0	143	
	7	0	50	
2	1	40	94	129.3 \pm 28.7
	3	80	162	
	5	90	139	
	13	326	122	
3	3	402	137	131.7 \pm 14.7
	9	45	115	
	14	92	143	
Sheep				
Uninfected	8	0	-2	9.0 \pm 12.2
	16	0	-1	
	34	0	8	
	40	0	3	
	53	0	30	
10 weeks post-infection	9	5	152	153.3 \pm 11.3
	11	41	150	
	12	59	157	
	29	133	172	
	30	153	152	
	41	80	137	

The results indicate that infections of cattle with adult *F.gigantica* are readily detected using the Pourquier test kit. The finding of high antibody levels in one animal without detectable adult flukes in the liver is in line with the findings with *F.hepatica* infected cattle, where antibody persistence after removal of flukes is reported. Although based on only a limited sample size, there does not appear to be a consistent relationship between numbers of adult flukes and antibody levels.

The finding of low negative S/P ratios in uninfected sheep, and consistent high antibody responses at 10 weeks after experimental infection indicates that the kit ELISA could also be used for sheep infected with *F.gigantica*, and probably also for *F.hepatica*.

More studies are clearly required but it appears promising to be able to use the kit for detection of fasciolosis in tropical countries, without having to modify it to make it specific for *F.gigantica*.

Objective 5: Time Course of Antibody Response and Sero-Conversion in Prepatent Infections.

Over 200 serum samples from a group of 10 calves each experimentally infected with 300 metacercaria *F.hepatica* were tested by both the Pourquier kit and the in-house ELISA. Samples were collected at approximately 2 to 4 week intervals for up to 78 weeks. Fluke egg counts had been conducted on faecal samples from some animals, but only from weeks 46 to the end of the trial. None of the cattle were treated with flukicide. The purpose of this component of the study was to determine the time at which positive antibody responses (sero-conversion) occurred in calves after infection, and if possible to determine from antibody responses at what point the fluke infections became mature and produced eggs in faeces.

The mean antibody levels as determined by the two methods are compared in Figure 6. As previously the positive cut-off was determined as 40 EU (in-house) and 30% S/P (Kit). Patterns of antibody detected by the two methods were very similar. The kit indicated sero-conversion had occurred before 2 weeks after infection (S/P 87%), and peak levels (>200% S/P) were recorded by 11 weeks, remained at high values until 20 weeks, before declining slightly over the remaining 54 weeks. Even after 18 months four out of five animals were positive.

Using the in-house test the sero-conversion was not evident until 6 weeks after infection, peaked between 11 and 24 weeks, declining gradually and becoming negative by week 68.

There was no apparent change in antibody levels, which could be attributed to patency of infection, that is there was no sudden elevation of antibody around the 12-14 week point at which time *F.hepatica* begins shedding eggs into the bile duct. Similarly the cessation of egg laying in some animals, in the period 46-68 weeks PI, was not correlated to significant drops in antibody, although there was a decline, with mean values becoming negative with the in-house assay.

7. 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.

Using natural infection in cattle with *Fasciola* in Vietnam, Anderson et al. (1999) claimed a sensitivity of 86% and 70% specificity for an ELISA that used an IgG conjugate against a crude excretory/secretory (ES) antigen. Bossaert et al. (2000) measured parasite-specific IgG1 and IgG2 isotypes in experimentally infected cattle against ES and somatic antigen fractions. IgG1 levels were much higher than the IgG2, and the IgG1 ELISA showed analogous sensitivity and specificity (92% and 94%) against both antigens. She used pre-determined cut-off values from contingency tables. The sensitivity results of our study indicated that the kit was highly sensitive, when the samples were collected from confirmed infection status naturally infected animals. Studies determining specificity and sensitivity based only on artificial infections should be regarded with caution.

No previous studies, other than unpublished data mentioned by Rolfe et al. (1993) indirectly implicating the presence of paramphistomes interfering with *F. hepatica* ELISAs have been conducted. Bossaert et al. (2000) who have examined some other cross reactivities against other helminths could not evaluate cross-reactions with either *Dicrocoelium* spp. (small liver fluke common in Europe, but absent in Australia) or *Paramphistomum* spp. due to the absence of monospecific sera.

As described above we experienced considerable difficulty in interpreting the kit ELISA results in the field infection studies with concurrent paramphistome infections. It was not possible to unequivocally discount the presence of *Fasciola*, and confirm that the positive ELISA results were due to paramphistomes infections only. The fact that the commonly used flukicides (triclabendazole, closantel, or clorsulon) have insignificant effectiveness against paramphistomes, and that *F. hepatica* antibodies persist for months after successful flukicide treatment, indicates that positive antibody results from the Pourquier kit on animals from areas with known concurrent infections should be examined carefully.

The fact that the history of the samples from the epidemiological study on paramphistomes in northern NSW was unclear has further complicated the study. The use of tracer calves of varying ages – and hence possible previous exposure to liver fluke as well as paramphistomes – means that the finding of sero-conversion and moderate to high paramphistome burdens may not be related. Low negative ELISA values in young animals with confirmed paramphistome egg counts and zero *F. hepatica* eggs supports the belief that paramphistome infection did not interfere with the kit results. Unfortunately, it is unlikely that any researcher will be able to prove this unequivocally by conducting artificial infections with paramphistomes into worm-free calves. There are no parasitologists currently working with *Paramphistomum* or *Calicophoron* in Australia, or have the capacity to maintain the parasite life cycles in the laboratory. This problem is unlikely to be resolved satisfactorily in the near future.

The lack of a marked decline in ELISA values recorded with the Pourquier kit following single treatment with triclabendazole (Figure 5), can be attributed to the lack of success of the treatment in removing all

the adult flukes. Our findings with the same material that Rolfe et al. (1993) used to suggest that antibodies persisted for 5 months after treatment casts some doubt on the accuracy of this period. However, other authors (Levieux et al. 1992) have shown that antibodies (measured in an haemagglutination assay) to the same F2 antigen used in the kit, were significantly lower from the second month after treatment in naturally infected cows treated with nitroxynil and oxcyclosanide one month later, than in an untreated control group. Negative values were reported 5-6 months after treatment (Levieux et al. 1992).

Castro et al. (2000) found that IgG antibodies were no longer detectable in a dot –ELISA by 4-6 months after animals received the first of three weekly doses of triclabendazole. They suggested that use of anti-IgM antibodies might overcome the problem by detecting *Fasciola* infections only in the active infection phase. Clearly the persistence of antibodies after incomplete removal of liver fluke is likely to be a limitation to using the kit as a means of confirming successful treatment, and hence its use as a method for testing stock to exclude animals from fluke-free areas such as Western Australia. There would therefore still appear a need for an antigen detection type of assay, which will only detect "active" fluke infections.

The finding of no interference with the *F.hepatica* kit ELISA from previous nematode infections is in line with few other studies. No cross-reaction with monospecific sera from animals infected with *Cooperia* or *Ostertagia* were detected (Bossart et al. 2000). These authors however, found that cross-reaction occurred to serum infected with several cestodes including *Echinococcus granulosus*, *Cysticercus tenuicollis* and *C. ovis*. This was in sheep serum, so the findings may be of limited relevance to cattle, where monospecific *C. bovis* infection did not cross react (Bossart et al 2000).

The capacity of the Pourquier kit to detect infection with *F. gigantica* in both cattle and sheep, is not of immediate importance to the Australian meat industry. However, it makes it a useful method for application in SE Asia, where either *F gigantica* occurs alone or in combination with *F.hepatica* (Anderson et al. 1999).

The ability of the kit to detect sero-conversion as soon as two weeks after infection, is a significant improvement over the in-house test where it is not until 6 weeks that clearly positive results are detected. This confirms the finding of Reichel (2002) using an earlier version of the kit. Bossart et al. (2000) also showed early elevation of IgG1 levels. These were more pronounced in single than trickle infections, and they suggested that juvenile and adult-stages produce cross-reacting antigens. It was possible there was a correlation between IgG1 responses and level of infection. As the Pourquier kit detects broad IgG responses and not the more specific IgG1 isotype, it is not possible to make similar deductions in the present case.

The general slow decline in antibodies after a long plateau phase, reported in both the time course study and the earlier flukicide treatment experiment, is similar to that reported by Bossart et al. (2000) and Reichel (2002) who found a minor decline after 6 weeks. Reichel also suggested that there was an increase in titre at about patency. Closer examination of his data suggests that this is not significant. Certainly, there was no significant jump in antibody in our studies, with either test method that could be attributed to maturity of egg-producing adult flukes.

Failure to detect any relationship between adult fluke numbers or faecal fluke egg counts and antibody readings in this project is similar to that reported by many authors (Reichel 2002, Bossart et al 2000, Hillyer 1999). This highlights another of the fundamental limitations of antibody detection methods no matter how purified the capture antigens are, or the specificity of the antibody isotypes.

8. OUTCOMES AND CONCLUSIONS

1. 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.
2. 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.
3. 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.
4. 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.
5. Limited studies with serum from animals infected with the related tropical liver fluke *Fasciola gigantica* indicate that the Pourquier ELISA kit may be used to detect this parasite in overseas countries.
6. The pattern of serological responses in cattle experimentally infected with *F.hepatica* was similar whether using the Pourquier Kit or the NSW Agriculture ELISA. IgG antibodies were detected earlier with the kit, and reached maximum levels sooner. Persistence of positive titres continued to at least 18 months in the absence of reinfection.
7. There was no apparent increase in antibodies detected by the kit that could be attributed to the maturity of adult liver fluke at the time of patency (production of eggs).
8. There was no relationship between positive kit results and either numbers of adult flukes or counts of fluke eggs in faeces.
9. The Pourquier ELISA kit is easy to use and is suitable for diagnostic parasitology laboratories equipped with an ELISA microplate reader.
10. 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 validation is conducted).
11. 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. The kit is not suitable for crush/race-side detection or as a barrier exclusion test.

9. ACKNOWLEDGMENTS

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Table 1. Comparison of Pourquier Kit ELISA with In-House ELISA in Sera from Tracer Weaner Cattle from the North Coast, NSW

Animal Nos.	Grazing Period*	Age-animals*	No. Tested	PreBleed		8 weeks		PreBleed		8 weeks	
				Kit Mean (S/P%)	±SD	Kit Mean (S/P%)	±SD	In-house # EU	±SD	In-house # EU	±SD
21-29	2	4-6 mos	9	77.6	47.8	110.2	73.5	40.6	31.4	40.9	24.1
30-39	3	3-5 mos	9	1.6	5.4	12.6	22	11.8	6.5	15.7	4.4
40-46	4	3-5 mos	7	64.0	65.5	72.7	59.5	26.3	26.5	36.0	25.6
51-59	5	5-7 mos	8	25.8	49.1	19.3	15.3	16.0	19.8	15.8	6.5
61-69	6	5-7 mos	9	8.3	8.9	8.6	9.8	6.6	4.4	18.4	10.3
81-89	8	5-7 mos	9	15.3	16.6	6.1	7.1	10.6	2.2	20.4	11.5
90-96	9	12-14 mos	7	15.7	27.0	6.5	9.6	17.8	14.1	18.7	5.1
101-109	7	6-8 mos	7	2.4	3.7	12.9	10.6	8.6	4.0	13.0	5.4

* Taken from Rolfe (1991)

Analysis courtesy of Dr D Palmer, Dept AG WA.

Figure 1. LPC Negative Property

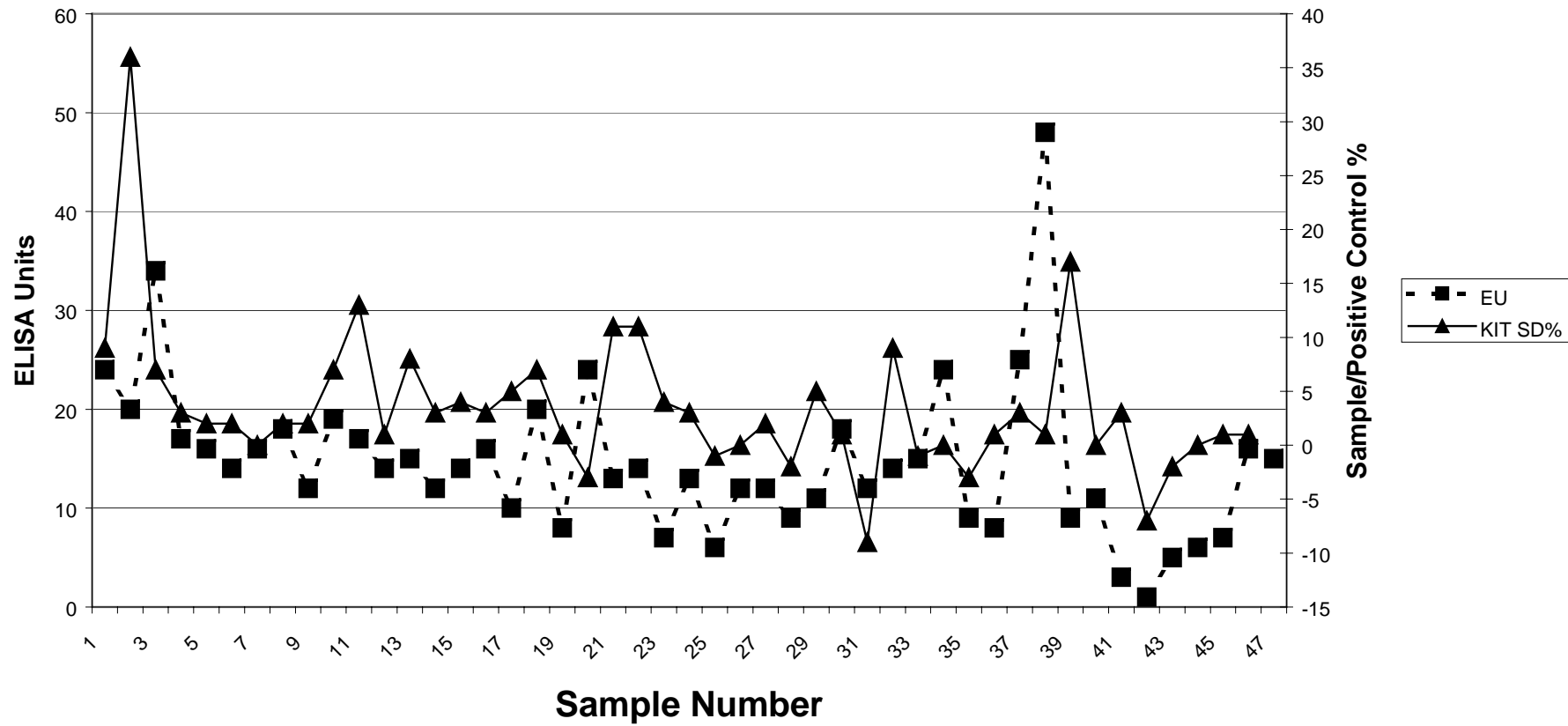


Figure 2. Endemic samples with matching faecal egg counts

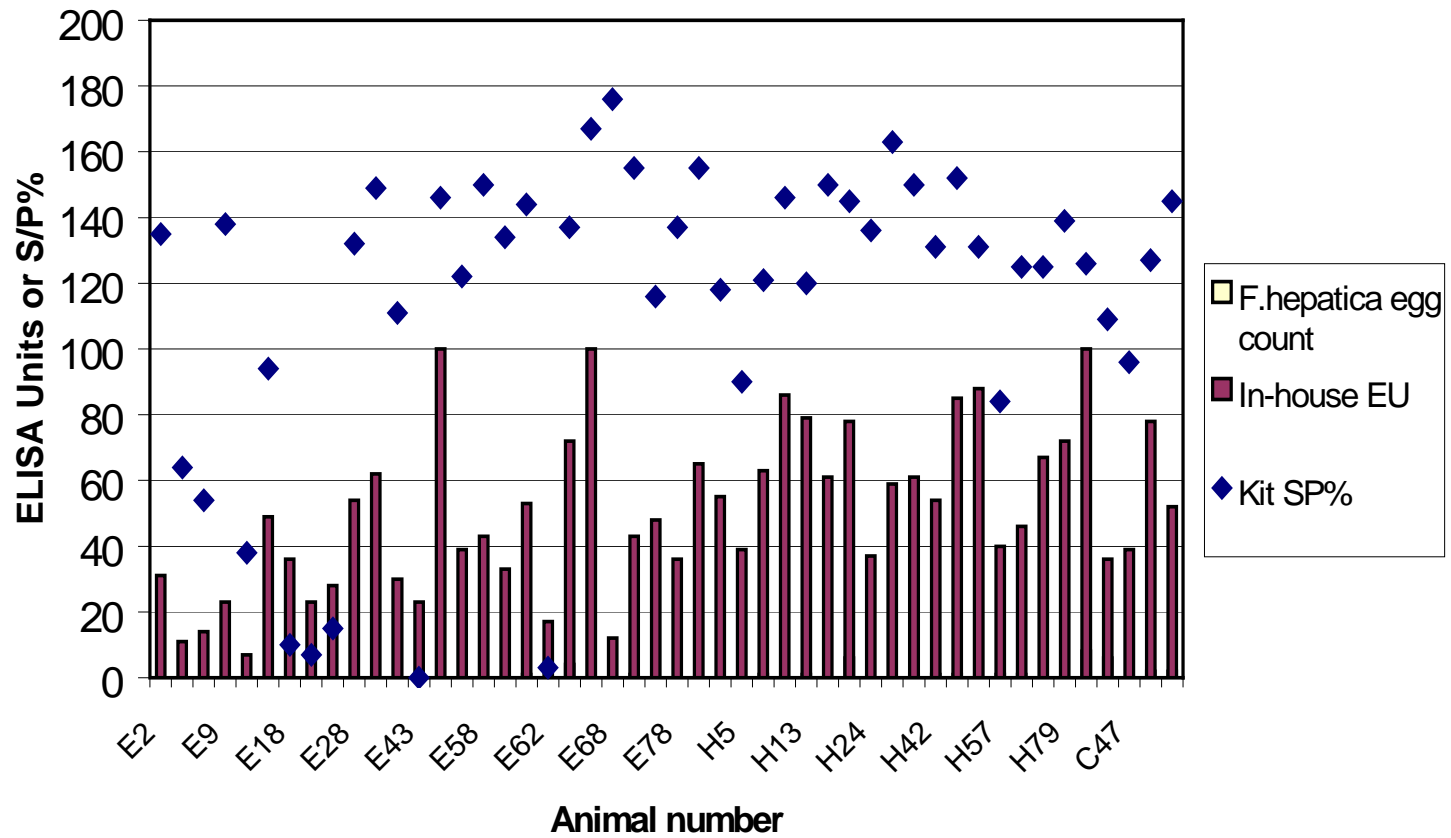


Figure 3. Paramphistome Epidemiology

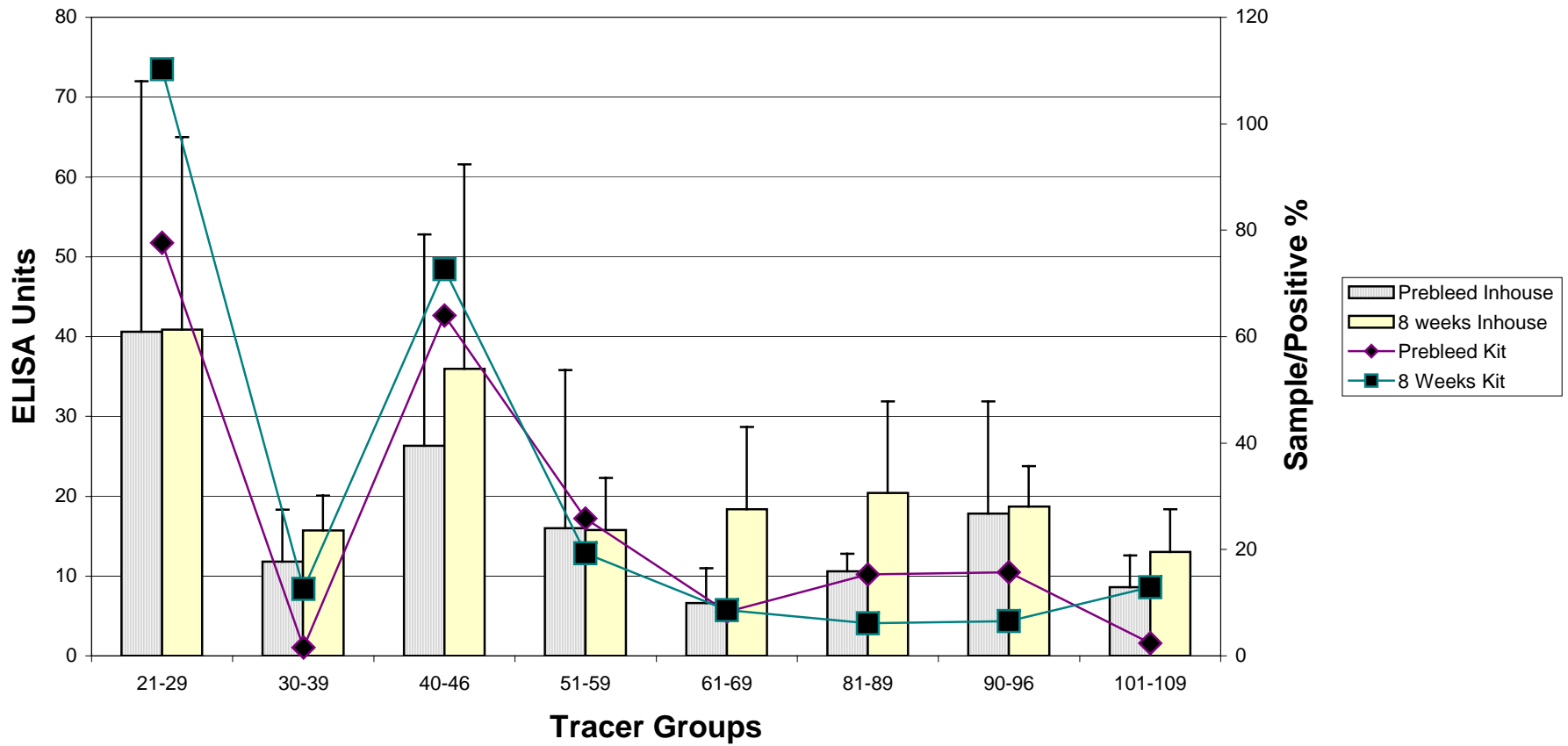
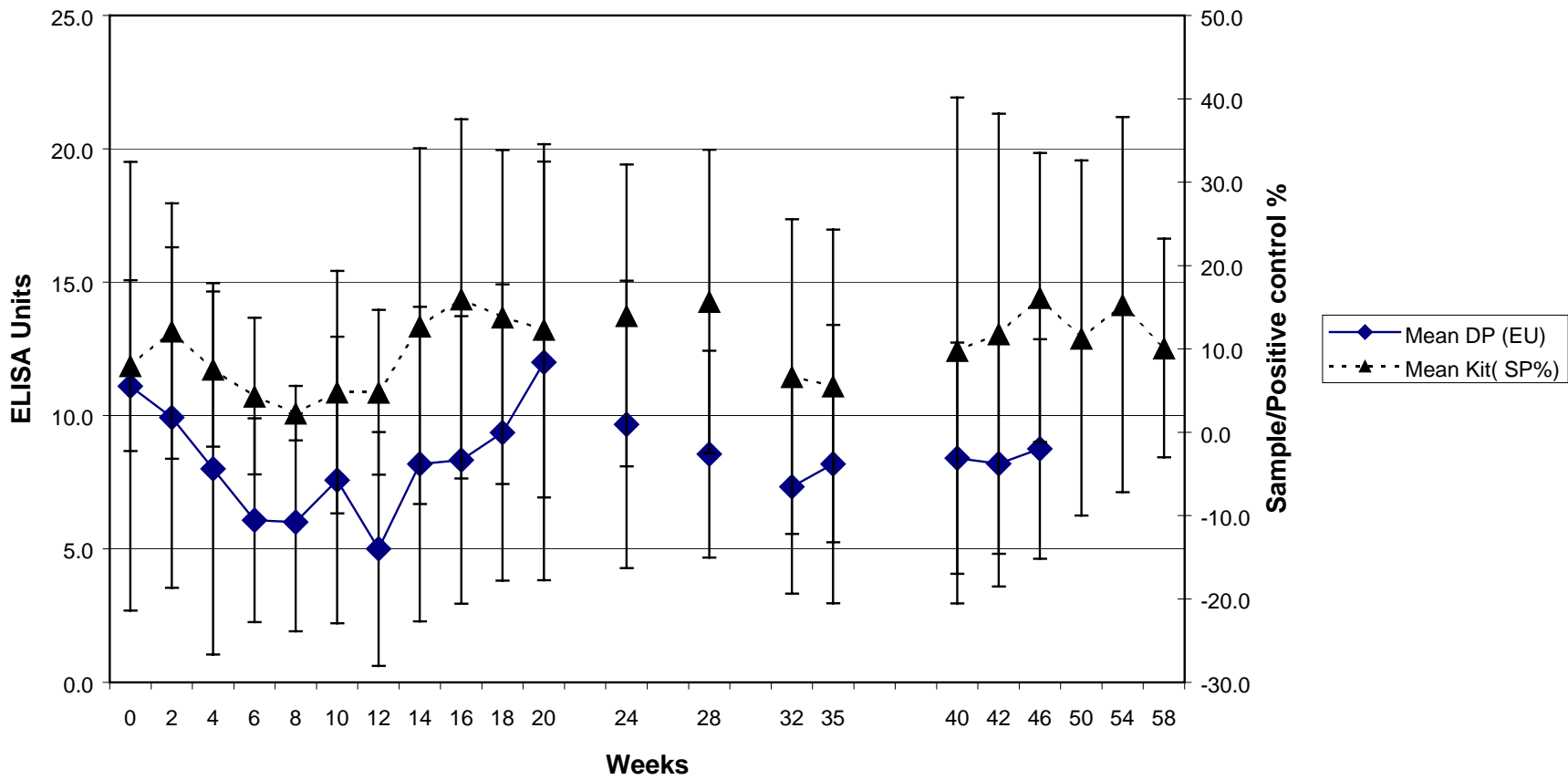


Figure 4. Comparison of Pourquier Fluke ELISA Kit with In-house ELISA
Non-infected Controls (Experiment PR10)



**Figure 5. Comparison of Pourquier Fluke ELISA kit with In-house ELISA
Infected animals (experiment PR10) and treated at week 40 with triclabendazole**

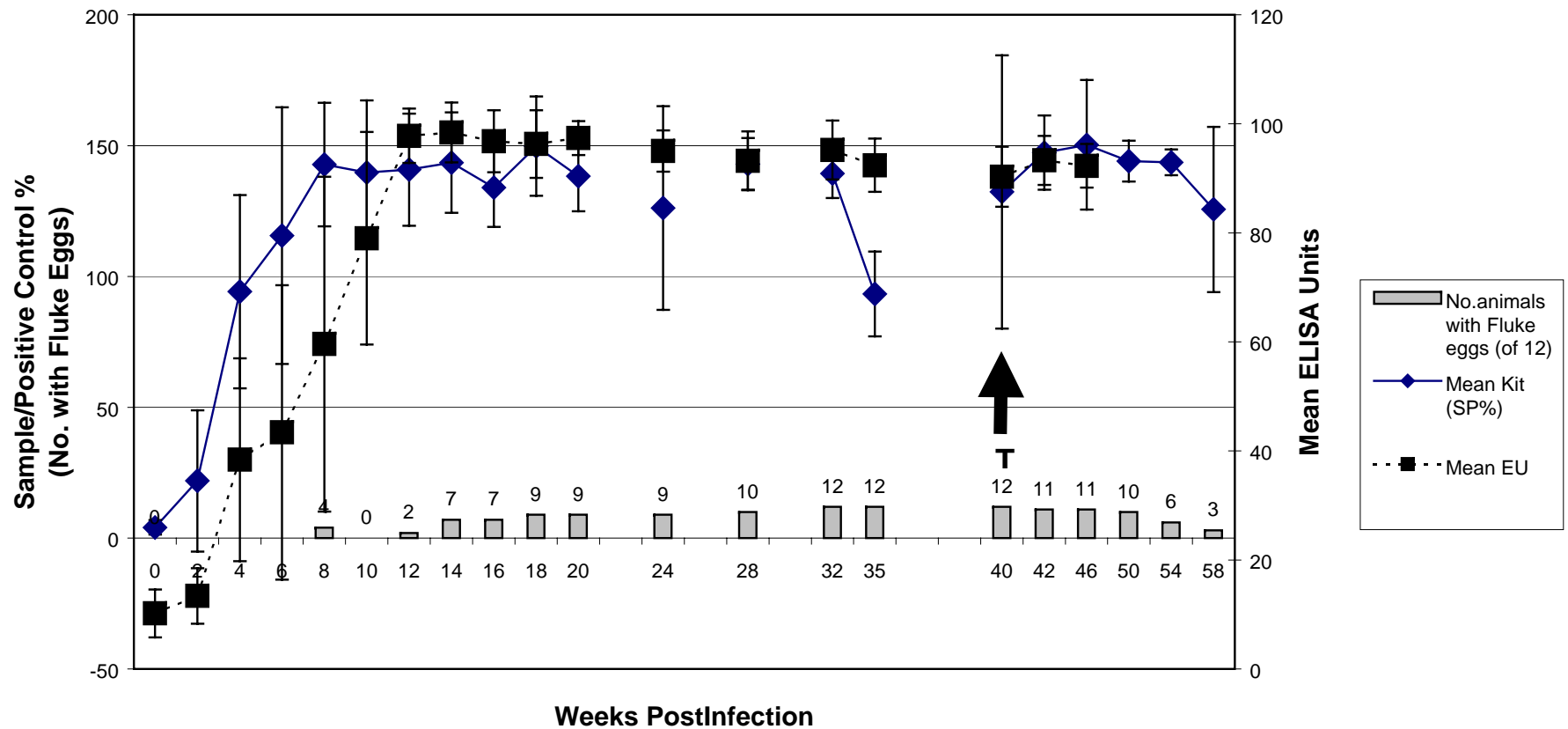


Figure 6. Time course of antibody responses measured by in-house ELISA (diamonds) and Pourquier Kit (solid squares) in 10 calves experimentally infected with 300 metacercariae of *Fasciola hepatica*

