

## DETECTION OF TREMATODE EGGS AND *EIMERIA LEUCKARTI* – SEDIMENTATION METHOD (FEST) – FAECAL SAMPLES

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### 1. Significance

Almost any animal species can be infected by trematode parasites. Trematode eggs are often too heavy to float in a flotation solution. Similarly, *Eimeria leuckarti*, a coccidian parasite of horses and donkeys is very heavy and can only be detected in sediments.

#### Coccidiosis

Infection with *Eimeria leuckarti*, a coccidian parasite of the small intestine of horses and donkeys, occurs throughout the world. However, it is a rarely reported infection because the oocysts do not rise to the surface on the usual flotation media and infection is often overlooked. The infection has only been reported twice in Australia, once in Tasmania and once in Victoria, but is believed to be much more common. The infection is usually symptomless, however, severe diarrhoea and deaths have been reported. The Department of Agriculture and Food Animal Health Laboratories have only seen this parasite once in more than 2000 horse samples examined for the presence of liver fluke infection.

#### Trematode infections

There are several important diseases of trematodes in domestic animals and the most important ones are briefly described below:

- **Fascioliasis**

Fascioliasis, the disease caused by infection with liver fluke, occurs in all herbivorous animals including pigs, kangaroos and humans. It is found in temperate high-rainfall regions all over the world, according to the distribution of freshwater snail intermediate hosts.

Recorded prevalence rates of liver fluke infection in cattle in endemic areas vary from about 10% in Europe to 95% in southern Latin America. Liver fluke infection in humans can reach a prevalence of 90% (the Lao People's Democratic Republic).

In eastern Australia, liver fluke infection occurs from south-eastern Queensland to southern Victoria and Tasmania. In South Australia, small endemic areas occur in the high-rainfall areas (irrigated pasture adjacent to the Murray river). In Western Australia outbreaks have occurred, but the parasite has not become established.

Fascioliasis is caused by flukes of the genus *Fasciola*. *F. hepatica* occurs in most countries and is the only species present in Australia. *F. gigantica* is the



more common liver fluke in Africa and Asia and *Fascioloides magna* infects wild and domestic ruminants in North America.

*F. hepatica* is a leaf-shaped, greyish-white parasite with one ventral sucker and can grow to 20 – 50 millimetres in length and 4 – 13 mm in width. *F. hepatica* is hermaphroditic and only one fluke is necessary to establish a patent infection. Each adult fluke can produce 20,000 eggs per day and may live for 10 years or more. Adults produce 200 times more eggs in sheep than in cattle where the egg production per adult fluke is only about 100 eggs per day. Eggs deposited in faeces hatch and release a miracidium, which infects freshwater snails. Cercaria develop and are released from the snail 4 to 7 weeks later. Cercaria encyst on vegetation as metacercaria, which are eaten by the host. The metacercaria excyst, immature flukes travel through the wall of the small intestine and migrate through the liver parenchyma to the bile ducts. They develop to egg-laying adult flukes in the main bile ducts about 8 – 10 weeks after infection.

The main intermediate hosts for *F. hepatica* are snails of the genus *Lymnaea*. *Lymnaea (Pseudosuccinea) columella* is an important intermediate host in many warm countries and until recently was the only intermediate host for *F. hepatica* present in Western Australia. *L. viridis* is an important intermediate host in New Guinea and in the Philippines, has been recorded in Brisbane (Queensland) and more recently also in Western Australia. *L. tomentosa* is an important intermediate host in Europe and also the eastern States of Australia.

Sheep may suffer severe liver damage and death is not uncommon. Affected animals are anaemic, hypo-proteinaemic, jaundiced and show signs of peritonitis. Fascioliasis in cattle is usually a chronic disease associated with low weight gains and decreased milk production. It can be the cause of significant economic losses through liver condemnation at slaughter.

Pathological changes due to migration of immature flukes are termed acute or subacute fascioliasis, and the response to adult flukes in the bile ducts, chronic fascioliasis. Both changes can be present in the same animals. Migratory flukes cause traumatic lesions to the liver parenchyma forming tortuous tracts that appear in cross sections as 2 – 3 mm diameter haemorrhagic foci. Microscopically, there is haemorrhagic necrosis infiltrated with eosinophils, histiocytes and giant cells. Repair of the lesion results in irregular hepatic fibrosis. In cattle, there may be massive fibrous thickening of the bile ducts in response to adult flukes. This is associated with cholangio-hepatitis and sometimes mineralisation. In most species, however, there is little thickening of the bile duct walls.

Diagnosis in individual animals relies on the demonstration of *F. hepatica* eggs in the faeces using the sedimentation technique. Eggs are thin-walled, golden yellow, have an operculum and measure 140 x 80 microns. They can be difficult to differentiate from paramphistome eggs. The sedimentation technique used in cattle only detects about 30% of all animals shedding eggs. Diagnosis of infection during the pre-patent period (8 weeks) is not possible.

- **Paramphistomiasis**

Intestinal paramphistomiasis caused by paramphistomes, or stomach flukes, is a severe debilitating disease of young cattle between 6 and 18 months of age.

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The clinical disease of severe ill-thrift and diarrhoea is caused by massive numbers of immature flukes that colonise the anterior small intestine causing severe damage to the intestinal mucosa. Adult flukes are found in the rumen and reticulum and generally cause no harm. The parasite has an indirect life cycle with planorbid snails as intermediate hosts.

## 2. Specimen (scope and application)

### 2.1 Scope

The test is a modified method based on Benedek (1943) and Boray & Pearson (1960). The test will detect the presence of trematode eggs or oocysts of the coccidian parasite *Eimeria leuckarti*. All stock being imported into Western Australia requiring a test before entry and after entry should be tested using this method (FEST) to adhere to WA's importation requirements.

The presence of trematode eggs does not always indicate the presence of live adult flukes and the absence of trematode eggs does not exclude trematode infection. Liver fluke and stomach flukes have a prepatent period of 8 weeks or more depending on the animal species. After successful treatment with a flukicide, liver fluke eggs present in the bile ducts and gall bladder can continue to be shed for 10 – 20 days.

### 2.2 Application

Faeces can be used as collected. Trematode eggs will not hatch if the temperature is below 10°C and can survive unhatched in the faeces for more than 6 months. Faeces can therefore be stored for months in the fridge prior to examination provided there is no overgrowth with fungus. However, they are susceptible to desiccation.

It takes 10 – 20 days for a miracidium to develop at a temperature of 16 – 20 degrees Celsius. Eggs only hatch if the temperature is above 10 degree, there is short wave light and the eggs are surrounded by fresh water and not in direct contact with faecal material. Faeces kept at room temperature for less than 10 days are still suitable for examination.

The number of liver fluke eggs shed in faeces can be low. Some species (e.g. cattle) can shed less than 1 egg/gram and, in horses, excretion rates as low as 0.1 egg/g have been reported. The procedure described here is based on 4 g (10g for horses and cattle) of faeces and the examination of the whole sediment. Sensitivity of the test is high (> 90%) if more than 10 eggs/g faeces are present. However, because lower egg counts are very common, the test is mainly recommended as a herd testing procedure. Overall, the sensitivity of the test is considered to be about 30 – 50%. If necessary, repeated analysis of 4 g samples can be conducted to improve sensitivity.

The test procedure is not suitable for the detection of eggs from the small liver fluke *Dicrocoelium dendriticum* (not known to occur in Australia). There is no reliable method for the detection of this parasite except for liver examination at the abattoir.



## 2.3 Sample preparation

No special sample preparation is necessary.

## 3. Quality Control

## 4. Principle

Trematode eggs and oocysts of *Eimeria leuckarti* are very dense and flotation in high-density (density > 1.3) media usually causes distortion of the eggs due to osmotic pressure. The higher density of the fluke eggs and oocysts of *Eimeria leuckarti* compared to faecal debris allows the separation of faecal debris from eggs. Faeces is filtered through two sieves to remove coarse debris, collected on a fine sieve and then processed through several rapid sedimentation steps. The more rapid sedimentation of the eggs separates them from the less dense faecal material. The supernatant is siphoned off and the whole sediment examined for the presence of trematode eggs or oocysts.

## 5. Reagent

(1). **0.5 % methylene blue:** Weigh 0.5 g of methylene blue stain into an appropriate beaker. Add 50 millilitres of deionised (DI) or reverse osmosis (RO) water and dissolve on a magnetic stirrer. Make up to 100 mL with DI or RO water and filter. Store in a dropper bottle at room temperature.

(2). **2% iodine solution:** Dissolve 100 g of potassium iodide and 20 g of iodine crystals in 1000 mL of DI or RO water. Dissolve the potassium iodide first, the iodine will then easily go into solution. (Use 12.5 mL per litre for positive egg solutions discards). Hold for 12 hours before discarding. This solution keeps indefinitely.

### CAUTION:

**Iodine** – highly irritating to skin, eyes and mucous membranes; harmful vapour; causes burns. Avoid contact with eyes and skin. Wear gloves and eye protection when making up iodine solution.

**Potassium iodide** – highly toxic intravenously; moderately toxic orally.

## 6. Equipment

- (1). **Dropper bottle.**
- (2). **Magnetic stirrer.**
- (3). **Sedimentation flasks** 250 mL or 500 mL. The vertical distance between the base of the flask and the 100 mL mark should not exceed 80 mm.
- (4). **Electronic balance**
- (5). **Spatula.**
- (6). **70 mL specimen jars** plastic.

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- (7). **Wash bottle.**
- (8). **Laboratory timer.**
- (9). **Vacuum pump**
- (10). **150  $\mu\text{m}$ , 90  $\mu\text{m}$  and 45  $\mu\text{m}$  mesh 20 cm diameter sieves** □
- (11). **Pasteur pipettes.**
- (12). **Pasteur pipette bulbs.**
- (13). **Glass petri dish (45 mm diameter) or other suitable viewing chamber e.g. Universal egg counting slides.**
- (14). **Inverted microscope** with measuring graticule or **stereo microscope** with measuring graticule.
- (15). **500 mL beakers**

## 7. Procedure

- (1). Weigh out 4 g (10g for horses and cattle) of faeces into a 500 mL beaker.
- (2). Add approximately 100 mL of tap water and thoroughly mix with a spatula to homogenise the faecal sample. Pre-soaking and vigorous stirring of firm faeces such as sheep, goats and alpaca may be necessary to aid in dispersion of the faecal pellet.
- (3). Set up the three sieves on top of each other with the 150  $\mu\text{m}$  mesh size on top, followed by the 90  $\mu\text{m}$  and 45  $\mu\text{m}$  mesh size sieves. The sieves should be placed on a surface that will not impede the free flow of water through the sieves, e.g. over a sink drain hole or corrugated sink drainer.
- (4). Pour the homogenised faeces through the sieves and follow through with a powerful jet of tap water from a suitable hose, until the faeces are thoroughly dispersed and the emergent water is clear. It may be necessary to partially separate the 90  $\mu\text{m}$  and 45  $\mu\text{m}$  sieves to break the air lock and prevent over-flow from the sieves.
- (5). Remove the 150  $\mu\text{m}$  sieve. Continue to wash through the 90  $\mu\text{m}$  and 45  $\mu\text{m}$  sieves, thoroughly dispersing the faecal material with the jet of water from the hose. Remove the 90  $\mu\text{m}$  sieve and repeat the above process with the 45  $\mu\text{m}$  sieve.
- (6). Incline the 45  $\mu\text{m}$  sieve at approximately 45° to the horizontal and gently wash the filtrate from the top to the bottom taking care not to spill any material. Tip the filtrate into a numbered sedimentation flask and gently rinse any residues from the sieve into the flask. Make up to the 100 mL mark with tap water and start the timer for 6 minutes.
- (7). Thoroughly reverse-rinse the sieves with a strong jet of water to prevent sample carry-over into subsequent samples (pay particular attention to the lip of each sieve, which may harbour some faecal material).
- (8). After 6 minutes has elapsed, suck off the supernatant from the sedimentation flask very carefully with the pump to the 20 mL mark. Ensure that the tube or pipette used to siphon off the supernatant remains close to the surface

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throughout the process, allowing an amount of air to be drawn into the tube, to avoid disturbing the sediment.

**NB:** If the sample is very dirty, then the sample can be split into two sedimentation flasks and combined again before transferring to the petri-dish.

- (9). Refill flask with water to 100 mL and repeat the above. The process can be repeated as many times as needed in order to clarify the sediment.
- (10). When the supernatant is clear, suck down to 10 mL and add one drop of methylene blue.
- (11). Leave for 5 minutes for effective staining of the debris.
- (12). Transfer all of the material into a viewing chamber or petri dish, ensuring all the sediment is included. View under an inverted microscope using 40x magnification. A dissecting microscope or a compound microscope can also be used providing the sample is cleared of floating debris by repeated sedimentation. A minimum magnification of 20x is suggested for reliable screening for the presence or absence of trematode eggs.
- (13). For all positive samples add 12.5 mL of 2% iodine solution per litre of discarded material and hold for 12 hours before disposal of wastes.

## 8. Results

Trematode eggs and coccidian oocysts remain unstained and stand out clear against the bluish stained vegetable matter.

### 8.1 Calculations

$$\text{Eggs/g faeces} = \frac{\text{Total number of eggs counted}}{\text{Total amount (in g) of faeces examined}}$$

### 8.2 Units

Eggs/g faeces

- 8.3** The oocysts of *Eimeria leuckarti* are 71 – 85 µm x 51 – 63 µm in size and have a 7 – 9 µm thick dark brown exterior shell with a rough surface. The interior sheath is colourless with a distinct micropyle.

*Fasciola hepatica* eggs are 140 µm x 80 µm, golden yellow with an indistinct operculum and the embryonic cells are also rather indistinct. They must be distinguished from the eggs of other flukes, especially the large eggs of paramphistomes which are of the same size. The paramphistomes are 120 – 180 µm x 70 – 90 µm (depending on species), have transparent shells and distinct opercula; their embryonic cells are clear and there is frequently a small knob at the posterior pole and they are pale brown to grey in colour.

Mite eggs can be of similar size as trematode eggs and are sometimes difficult to differentiate from liver fluke eggs. They are usually darker yellow, orange or even brown and slightly asymmetrical with very distinct embryonic cells. Most mite eggs have no operculum, but some do. The operculum, when present, is very distinct, and usually well within the upper third of the shell.

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## 9. Validation

Modified technique, validation report attached (Appendix 1).

## 10. Reference Ranges

N/A

## 11. Reporting

Reported only as positive or negative for *Fasciola hepatica*, paramphistomes or *Eimeria leuckarti* (the former *only* is required for regulatory import testing of animals into WA). Number of eggs/oocysts/g faeces only provided on request.

**Other laboratories using this method for testing animals entering or having already entered into WA as per import requirements are required to state on their laboratory report that this DAFWA method has been adhered to.**

## 12. Notes

The presence or absence of *Fasciola hepatica* is a significant finding because Western Australia is free of liver fluke infection. For this reason the results are reported as positive and negative only. The presence of other trematode eggs or of oocysts of *Eimeria leuckarti* is, in most instances, without clinical significance, but is reported or referred to in the 'Comments' section.

## 13. Glossary of Terms

## 14. References

4450 **Benedek L** (1943). Untersuchungen auf Leberegeleier durch Sedimentation. *Allatorv. Lap.* 66: 139–141.

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## Appendix 1:

### Validation report:

# VALIDATION OF THE FAECAL SEDIMENTATION METHOD (PAM-26) FOR THE DETECTION OF *FASCIOLA HEPATICA* EGGS IN FAECES FROM CATTLE AND HORSES:

Jeff Mitchell and Dieter Palmer

## FINAL REPORT

### Introduction

Egg counts for the detection of liver fluke infection are routinely conducted in many laboratories. Because liver fluke infection is common in most parts of the world, methods for the detection of liver fluke eggs in faeces have concentrated mainly on the quantitative aspect of the assessment. Surprisingly, very few studies have validated the technique and to our knowledge only one study has attempted to determine the detection limit of the test.

The most commonly used technique is a sedimentation method described by Boray (1960) and later modified by Happich and Boray (1969). They found that the technique was able to detect eggs in all samples with more than 10 eggs/gram using 3 g of sheep faeces. The percentage of recovered eggs was approximately 40%. Faeces with less than 10 eggs/g were not tested.

Breza and Corba (1973) compared a flotation-sedimentation method with the sedimentation method of Happich and Boray (1969) and found that in an analysis of 120 naturally infected animals, the flotation-sedimentation method had a sensitivity of 100% compared to 92% for the sedimentation method. The flotation-sedimentation method recovered up to 10 times more eggs in the samples than the sedimentation methods. However, the actual number of eggs present in the samples was unknown.

More recently a glass bead sieving method has been described for the detection of *Fasciola hepatica* eggs in faeces (Taira, 1985). The test was able to detect eggs in all samples that contained at least 8 eggs/g faeces. The test examines only one gram of faeces. At a faecal egg concentration of one egg/g only 55% of all samples were positive.

The parasitology laboratories at the Department of Agriculture and Food Western Australia use an in-house modification of the sedimentation method described by Boray (1960) and Happich and Boray (1969). A validation of our test method is important because we rely on the test to prevent liver fluke infected animals from entering WA.

### Methods

Sheep faeces containing large number of *Fasciola hepatica* eggs were obtained from NSW Agriculture (courtesy of Dr Joan Lloyd and Dr Gareth Hutchinson). Eggs were purified using large-scale sieving followed by sedimentation.

Faeces from cattle and horses free of liver fluke infection were obtained and 4 g or 10 g of faecal samples were spiked with a known number of *F. hepatica* eggs. The samples were examined following our standard sedimentation technique (PAM-26), except that in all but one series of samples the use of the 90 µm sieve was omitted and only the 150 µm and the 38 µm sieve were used.

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## Results

The results are shown in Table 1. The detection limit for the examination of 10 g of faeces from bovines or equines was 1 egg/g faeces or ½ egg/g respectively. If only samples of 4 g of faeces were examined (cattle only), the detection limit was 10 eggs/g faeces. The percentage of eggs recovered varied from 0% to 68% depending on the total number of eggs present in the sample.

Table 1: Recovery of trematode eggs and detection limit in faeces from cattle and horses.

Species	Sample amount	Total number of eggs in sample (eggs/g)	Number of samples tested	Number of positive samples	Number of eggs recovered max-min	Average % recovery
Bovine	4 g	40 (10 eggs/g)	10	10	16–39	58%
Bovine	4 g	10 (2.5 eggs/g)	2	1	0–3	15%
Bovine	4 g	8 (2 egg/g)	10	4	0–2	6%
Bovine	4 g	6 (1.5 egg/g)	10	3	0–2	7%
Bovine	4 g	4 (1 egg/g)	10	6	0–1	15%
Bovine	4 g	2 (½ egg/g)	10	2	0–1	10%
Bovine	10 g	10 (1 egg/g)	10	9	1–3	12%
Bovine	10 g*	10 (1 egg/g)	10	10	1–5	23%
Equine	10 g	500 (50 eggs/g)	6	6	170–338	68%
Equine	10 g <sup>#</sup>	20 (2 eggs/g)	10	10	1–5	14%
Equine	10 g	10 (1 egg/g)	10	9	0–4	18%
Equine	10 g	5 (½ egg/g)	10	10	1–3	24%
Equine	10 g <sup>#</sup>	3 (⅓ egg/g)	10	0	0	0%

\* used additional 90 µm sieve (see PAM-26)

# different batch of horse faeces

The inclusion of the additional 90 µm sieve did not decrease the sensitivity. Care has to be taken though to prevent overflow of the sieve when the samples are sieved through the series of sieves.

## Discussion

Sheep are very good hosts for *F. hepatica* and tend to shed a large number of eggs. This is not only an effect of the dilution factor but also because adult liver fluke produce about 200 times more eggs in sheep than in other species (i.e. up to 20,000 eggs per day). This makes the sheep a very good host for liver fluke. The sensitivity of the detection method for this species is therefore not very critical and was not included in this validation. The number of eggs in faeces of cattle and horses on the other hand can be very low. In horses with *F. hepatica* infection as few as 3 eggs in 10 g have been reported.

The validation showed that at least one egg/g (½ egg/g for horses) can be detected with our technique provided that a faecal sample of 10 g faeces is examined. However, using

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4 g of faeces for cattle (current protocol) the detection limit was only at 10 eggs/g. This indicates that the total number of eggs present in the sample is more critical for the sensitivity of the technique than the egg concentration. Egg recovery in larger samples is probably lowered but the detection limit is still better.

The detection limit for the glass bead sieving technique described by Taira (1985) was 4 eggs/g using a sample of 1 g of faeces only. It is possible that this detection limit could be improved if the glass bead technique could be adapted to the processing of larger sample sizes.

### Recommendation

We recommend that the currently used sedimentation method for the detection trematode eggs be modified to change the sample size for cattle from 4 g to 10 g.

All three sieves (150  $\mu\text{m}$ , 90  $\mu\text{m}$  and 45  $\mu\text{m}$ ) should be used for sample preparation (no change of method). However, extreme care has to be taken to avoid overflow of sample material.

We also recommend that all laboratories conducting testing for the presence of liver fluke in WA be tested for their ability to detect liver fluke eggs in samples spiked with eggs at the detection limit of the assay every 6 months (QA control).

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