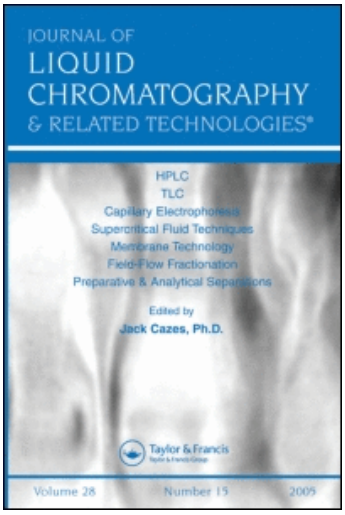


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# High Performance Thin Layer Chromatographic Analysis of Neutral Lipids in the Feces of BALB/c Mice Infected with *Echinostoma caproni*

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**Abstract:** High performance thin layer chromatography was used to determine neutral lipid profiles in the feces of BALB/c mice infected with adults of *Echinostoma caproni* (Trematoda). An approximate 25 worm burden per host induced changes in the neutral lipid profile of the mice at 1 to 5 weeks post infection (PI) relative to the uninfected controls. Infection caused a significant decrease (Student's *t*-test, $P < 0.05$) in the triacylglycerol fraction at 3 weeks PI and a significant increase (Student's *t*-test, $P < 0.05$) in the free sterol fraction at 2 weeks PI relative to the uninfected controls. Neutral lipid profiles of feces may serve as an indicator of infection by intestinal trematodes in animals and humans.

**Keywords:** Thin layer chromatography, TLC, Neutral lipids, Mice, *Echinostoma caproni*, Trematoda

## INTRODUCTION

The intestinal trematode *Echinostoma caproni* provides a good model to study intestinal trematodiasis in a murine host.[1] Information obtained from this

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model may be helpful in providing a better understanding of intestinal trematodiasis in humans. Human infections with intestinal trematodes are a global problem with reports of many cases per year in both developed and undeveloped countries. Most cases of intestinal trematodiasis result from eating tainted food products (fish, frog, snake, and invertebrates) infected with the metacercarial, or cyst stage, of various trematodes. Diagnosis of these trematode infections is based on recognizing characteristic eggs in the feces of infected animals or humans. Egg diagnosis from stool samples is tedious and time consuming and requires considerable skill on the part of the examiner.

Recent work has involved diagnosis by metabolic profiling and has mainly been done in mice infected with *Schistosoma mansoni*. In such studies, urine or feces from infected hosts is examined for certain analytes that might appear in significantly different concentrations in the urine or feces of infected hosts versus control hosts. In the one published study to date with mice infected with *S. mansoni*, Wang et al.[2] found a reduced level of certain tricarboxylic acid cycle intermediates in the urine of infected mice relative to the uninfected controls.

In view of the simplicity of the *E. caproni*-mouse model, we have begun metabolic profiling studies to determine if there are differences in certain analytes in the feces and urine of mice infected with *E. caproni*. The purpose of this study was to determine the effects of *E. caproni* infection on the neutral lipid content of feces of BALB/c mice using high performance thin layer chromatography (HPTLC).



## EXPERIMENTAL

### Mice Maintenance and Infection

BALB/c male mice, 6–8 weeks old and each weighing 20–25 g, were used as experimental hosts for *E. caproni* infection. Six mice were individually exposed per os to approximately $50 \pm 10$ metacercarial cysts of *E. caproni*. The six infected mice, marked with an ear punch, were housed together in a plastic mouse cage ($15 \times 27 \times 14$ cm) and fed Mazuri rodent food (PMI Nutrition, Henderson, CO, USA) and water ad libitum. The six control mice were handled and treated identically except they were not exposed to *E. caproni* and thus remained parasite free throughout the study. Feces were collected from individual mice on a weekly basis beginning at 1 week post-infection (PI) up to 5 weeks PI. Previous work with this model showed that mice exposed to this dosage of *E. caproni* cysts had about $25 \pm 10$ sexually mature adults of *E. caproni* in the small intestine by 2 weeks PI.[3] Previous work also indicated that *E. caproni* infection was long-lived in mouse hosts with worms remaining in the intestine for at least 2 months PI.[3]

## Sample Preparation

To collect feces, mice were removed from their cages and individually placed in plastic circular containers (15 cm diameter × 18 cm height) lined with filter paper on the bottom. Fecal pellets were collected for each sample (approximately 120 mg of feces per sample) after 30 min of isolation. Such collections were made from each of the six infected and six uninfected mice weekly from 1 to 5 weeks PI. Light microscopy of conventional fecal smears on glass slides prepared from the feces of the exposed mice beginning at 2 weeks PI showed that all mice were infected based on the presence of characteristic *E. caproni* eggs.[1] Such eggs were released from the sexually mature worms into the mouse feces by 10 days PI. All six infected hosts were necropsied at the end of the experiment and yielded $25 \pm 10$ worms per host.

To determine if isolated eggs may contribute to the neutral lipid profile of the mouse feces, about 1000 eggs (<2 mg blotted dry weight) were dissected from the uteri of five worms at 5 weeks PI. These eggs were extracted in chloroform-methanol (2:1) and prepared for chromatography as described below for lipid extraction from feces.



## Lipid Extraction

Feces were homogenized using a 7 mL capacity Wheaton (Millville, NJ, USA) glass homogenizer. The lipids were extracted in chloroform-methanol (2:1) in a ratio of 20 parts of solvent to 1 part feces. In order to completely extract the lipids, first the feces were homogenized in approximately 4 mL of chloroform-methanol (2:1) and the extract was filtered through cotton into a glass vial. Next, extraction was performed again with approximately 2 mL chloroform-methanol, and that extract was again filtered through cotton into the vial containing the first extract to form one sample. If any feces remained in the homogenizer, a third extract was obtained in the same manner as the second extract and combined in the same vial as one sample. Then, with a ratio of 4 parts sample volume to 1 part salt solution, Folch et al.[4] wash (0.88% KCl, w/v, in deionized water) was added to the vial and vortex mixed, and then the top, aqueous layer was removed and discarded. The samples were evaporated to dryness in a warm water bath (40–60°C) under nitrogen gas and then reconstituted in chloroform-methanol (2:1). The reconstitution volume was chosen so that the densitometric area of zones in sample chromatograms were bracketed within the scan areas of the standard zones and was typically 200–400 μL. The samples may be stored for later use at multiple stages: the feces for 24 h in a refrigerator at 0°C, the extracted lipids in chloroform-methanol (2:1) and the dried lipids for a year in a freezer at −20°C, and the reconstituted lipids for two days in a freezer at −20°C.

## Thin Layer Chromatography

The standard for neutral lipid analysis was the TLC Reference Standard 18-4A (Nu-Check-Prep, Elysian, MN, USA), which contained 20.0% each of cholesterol (CH), oleic acid (OA), triolein (TR), methyl oleate (MO), and cholesteryl oleate (CO). The standard zones were used to represent free sterols (FS), free fatty acids (FFA), triacylglycerols (TG), methyl esters (ME), and steryl esters (SE), respectively, in the samples. The solid standard was weighed into a 25 mL volumetric flask and diluted with chloroform-methanol (2:1) to prepare a standard solution containing 0.207 $\mu g\ \mu L^{-1}$ for each of the components.

HPTLC analysis was performed on either LHPKDF silica gel plates (Whatman, Florham Park, NJ, USA) or silica gel 60 $CF_{254}$ plates (EMD Chemicals Inc., an affiliate of Merck KGaA, Darmstadt, Germany). Both of these HPTLC plates are $10 \times 20$ cm and contain 19 scored lanes and a preadsorbent spotting area. Plates were precleaned by development to the top with dichloromethane-methanol (1:1). The standards (2.00, 4.00, 8.00, and 16.00 $\mu L$) and reconstituted samples (2.00, 4.00, and 8.00 $\mu L$) were applied to the preadsorbent zone in individual lanes with a 10- $\mu L$ Drummond (Broomall, PA, USA) digital microdispenser.

Plates were developed with the Mangold[5] mobile phase, petroleum ether-diethyl ether-glacial acetic acid (80:20:1), for a distance of 8.0 cm past the preadsorbent-silica gel interface in a Camag (Wilmington, NC, USA) twin-trough chamber containing 25 mL of the mobile phase in each trough and a saturation pad (Analtech, Newark, DE, USA) in the trough opposite to the one used for development. The development time was 8–9 min. Developed plates were dried in a fume hood with cool air from a hairdryer for 5 min, sprayed with 5% ethanolic phosphomolybdic acid solution, and heated for 10 min at 115°C on a Camag plate heater until blue neutral lipid zones appeared on a yellow background.

Quantitative densitometric analysis was done with a Camag TLC Scanner II using the tungsten light source set at 610 nm. The settings were slit width 4, slit length 4, and scanning speed 4 mm/s. The CATS-3 software was used to create a linear regression calibration curve relating the weights of the standard zones (0.414–3.31 $\mu g$) to their peak areas. If the area of more than one aliquot of a particular sample was bracketed within the calibration curve, the weight interpolated from the calibration curve corresponding to the sample area closest to the areas of the middle two standards was used to calculate the percent of the lipid.

The percent weight of lipid in fecal samples was calculated by multiplying the interpolated compound weight by the ratio of the reconstitution volume to the volume of sample spotted, dividing by the wet weight of the sample ($\mu g$), and multiplying by 100.

Student's *t*-test was used to determine the significance of data based on the mean $\pm$ standard error values of the lipids of a sample population at 1 to 5 weeks PI, with $P < 0.05$ being considered significant.

## RESULTS

Neutral lipid standard zones yielded $R_F$ values for CH, OA, TR, MO, and CO of 0.21, 0.34, 0.57, 0.66, and 0.79, respectively, in the Mangold mobile phase. FS, FFA, and TG were identified in the fecal samples based on comigration of their zones with the corresponding standard zones. ME and SE were not unequivocally identified in the fecal samples based on their differential migration compared to the MO and CO standards in the Mangold mobile phase. These fast-moving zones were not further characterized in this study.

As seen in Figure 1, dense zones (labeled X on the chromatograms) in the samples from infected feces (lanes 5–10) were seen at an $R_F$ value of 0.26; these zones were considerably less dense in feces from uninfected hosts (lanes 11–16). According to data in Kates,[6] these zones between FS and FFA in the Mangold mobile phase are probably diacylglycerols (DG). Since a DG standard was not used in this study, these zones were not further characterized. These unidentified zones (labeled X) were also seen clearly in Figure 2 between the FS and FFA zones.

Figure 1 shows qualitative differences in several fractions of neutral lipids in infected versus uninfected fecal samples. Of particular interest in the chromatograms is the apparent increase in the FFA and FS fractions in infected (lanes 5–10) versus uninfected (lanes 11–16) feces. Quantitative data (Table 1) show that differences in FS were significantly greater at 2 weeks PI for infected versus uninfected samples. Also, although not

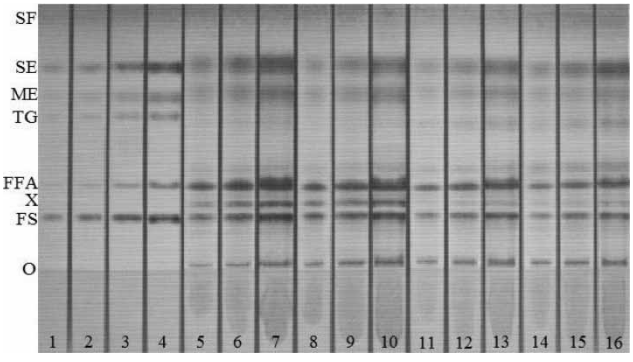


***Figure 1.*** Chromatograms on an EMD HPTLC plate photographed in white light with a Camag VideoStore documentation system plate showing the increase in free sterols in 2 samples of feces from infected BALB/c mice (lanes 5–10) versus 2 samples of feces from uninfected BALB/c mice (lanes 11–16) at 5 weeks post infection. Each sample was applied as 4, 8, and 16 μL. Lanes 1–4 contain the neutral lipid standard spotted at 2, 4, 8, and 16 μL, respectively. SF = solvent front, SE = steryl esters, ME = methyl esters, TG = triacylglycerols, FFA = free fatty acids, X = unknown zone, probably diacylglycerols, FS = free sterols, O = origin.

significantly different, the FFA fraction was about 1.5 times greater in the infected versus uninfected samples at 5 weeks PI.

The marked difference in the TG fraction between infected and uninfected samples can be seen in the chromatograms in Figure 2. The TG zones were completely absent in the infected samples (lanes 5–10) though visible in the uninfected samples (lanes 11–16).

Table 1 presents data for the weight percent of FS, FFA, and TG in the feces of infected versus uninfected samples at 1 to 5 weeks PI. There was a significant increase in FS at 2 week PI while there was a significant decrease in TG at 3 week PI.

HPTLC analysis failed to detect neutral lipids in the isolated egg samples containing approximately 1000 eggs, indicating that egg lipids do not contribute to the neutral lipid content observed in the feces.

## DISCUSSION

Worm infections of about 25 per host induced some significant changes in the neutral lipid profiles of the feces of BALB/c mice infected with *E. caproni*. Thus, the TG fraction was significantly reduced at 3 weeks PI and the FS fraction was significantly increased at 2 weeks PI in infected versus uninfected mice. Our findings show that neutral lipid profiles may serve as indicators of infection in mice with *E. caproni*. Thus, examination of neutral lipid fecal profiles by HPTLC may be helpful as a screen to supplement classical fecal examinations to detect echinostome infections in mice. The implication of

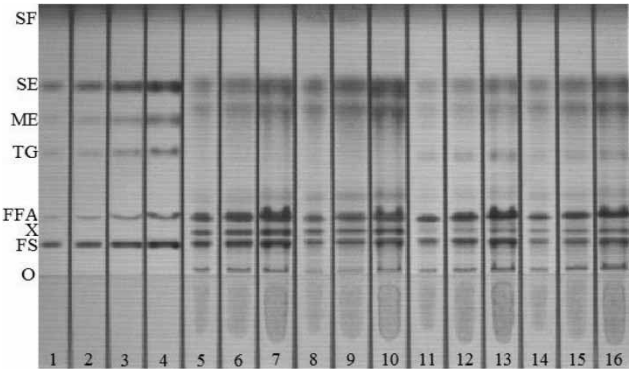


***Figure 2.*** Chromatograms on an EMD HPTLC plate photographed in white light with a Camag VideoStore documentation system plate showing the decrease in triacylglycerols in 2 samples of feces from infected BALB/c mice (lanes 5–10) versus 2 samples of feces from uninfected BALB/c mice (lanes 11–16) at 5 weeks post infection. Each sample was applied as 4, 8, and 16 μL. Lanes 1–4 contain the neutral lipid standard spotted at 2, 4, 8, and 16 μL, respectively. See Figure 1 for abbreviations.

***Table 1.*** Percent by weight (mean ± SE[a]) of neutral lipids in feces of BALB/c mice infected with *E. caproni* at 1 to 5 weeks PI

| Week post infection | Free sterols | | Free fatty acids | | Triacylglycerols | |
|---|---|---|---|---|---|---|
| | Infected | Uninfected | Infected | Uninfected | Infected | Uninfected |
| 1[b] | 0.260 ± 0.072 | 0.123 ± 0.032 | 1.15 ± 0.30 | 1.27 ± 0.20 | 0.365 ± 0.18 | 0.390 ± 0.20 |
| 2[c] | 0.273 ± 0.044[d] | 0.123 ± 0.032 | 3.67 | 1.27 ± 0.20 | 0.228 ± 0.096 | 0.390 ± 0.20 |
| 3[b] | 0.260 ± 0.027 | 0.239 ± 0.10 | 3.27 ± 1.5 | 2.68 ± 1.3 | 0.0466 ± 0.0081[e] | 0.177 ± 0.044 |
| 4[c] | 0.357 ± 0.12 | 0.194 ± 0.049 | 1.61 ± 0.67 | 2.73 ± 0.74 | 0.120 ± 0.050 | 0.260 ± 0.069 |
| 5[c] | 0.558 ± 0.33 | 0.217 ± 0.10 | 1.82 ± 0.61 | 1.24 ± 0.18 | 0.0301 | 0.400 ± 0.10 |

[a]SE = standard error.
[b]Sample size = 3–5.
[c]Sample size = 1–5; where no SE reported, n = 1.
[d]Value significantly increased compared to uninfected samples (Student's *t*-test, $P < 0.05$).
[e]Value significantly decreased compared to uninfected samples (Student's *t*-test, $P < 0.05$).

our finding is that neutral lipid profile changes as determined by HPTLC may be helpful in monitoring worm infections in animals and humans infected with intestinal trematodes.

The present study used a moderate infection of 25 worms per host based on an initial inoculum of about 50 cysts per host. As reported previously by Balfour et al.[7] mice can be infected with a high dose of *E. caproni* (about 100 cysts per mouse) to yield about 50–75 worms per host. Such infections, considered "heavy" by Balfour et al.,[7] may increase morbidity and mortality in the mouse but may also produce a more marked effect on the neutral lipid profile of infected versus uninfected hosts. Further studies on the neutral lipid profiles of feces of mice heavily infected with *E. caproni* are warranted.

We have found a large unidentified neutral lipid fraction in the feces of both uninfected and infected hosts, and have tentatively referred to it as DG based on data in Kates.[6] Further studies using DG standards to determine the concentrations of these glycerols in the feces of infected versus uninfected mice are needed.

Lastly, a previous study by Rivas et al.[8] reported certain differences in blood and tissue neutral lipids in ICR mice infected with *E. caproni*. That study was interventional, meaning that the mice were necropsied to obtain tissues and blood for comparative neutral lipid analysis of infected versus uninfected hosts. Rivas et al.[8] showed significant differences in FS and FFA concentrations in tissue and blood in uninfected versus uninfected hosts. That study was important in determining pathobiochemical differences in mice infected with *E. caproni* versus controls. Because the Rivas et al.[8] study was interventional, it was not appropriate for metabolic profiling where the collection of samples from the host must be non-interventional to make the profile studies practical.



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