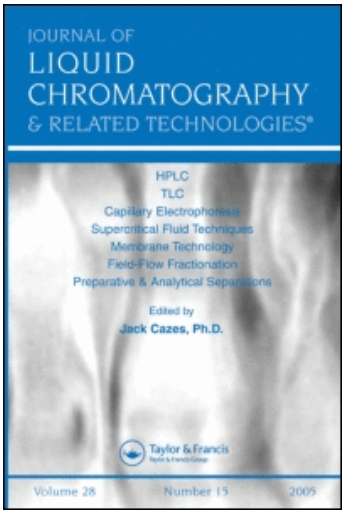

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Further Studies on the Neutral Lipid Content in the Feces of BALB/c Mice Infected with *Echinostoma caproni* as Determined by Silica Gel HPTLC-Densitometry

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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 3 to 10 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 7 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: *Echinostoma caproni*, Mice, Neutral lipids, Thin layer chromatography, TLC, Trematoda

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INTRODUCTION

The intestinal trematode *Echinostoma caproni* provides a good model to study intestinal trematodiasis in a murine host.[1] Information obtained from this 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* and *E. caproni*. 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 a previously published study 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. Following that study, Wang et al.[3] found that *S. japonicum* infection in hamsters caused a reduction in certain urinary tricarboxylic acid cycle intermediates, including citrate and succinate, and an increase in pyruvate relative to the amount in uninfected hosts. In a recent study with mice infected with *E. caproni*, Bandstra et al.[4] found a reduced level of triacylglycerols and an increased level of free sterols in mouse feces relative to the uninfected controls at 2 to 5 weeks post-infection (PI). Murray et al.[5] found no significant changes in phospholipid or sphingolipid concentrations in feces of BALB/c mice infected with *E. caproni* relative to the uninfected controls. Finally, Vasta et al.[6] found that the urine from BALB/c mice experimentally infected with *E. caproni* contained significantly increased levels of methyl esters at 6 and 7 weeks PI relative to the uninfected controls.

Because of the simplicity of the *E. caproni*-mouse model, we have continued metabolic profiling studies performed by Bandstra et al.[4] to determine if there are further differences in certain analytes in the feces of BALB/c mice infected with *E. caproni*. Therefore, the purpose of this study was to determine the effects of *E. caproni* infection with an approximate 75 metacercarial cyst exposure per mouse on the neutral lipid content of feces of BALB/c mice from 3 to 10 weeks PI using high performance thin layer chromatography (HPTLC) with quantification by densitometry.

EXPERIMENTAL

Mice Maintenance and Infection

Mixed gender BALB/c 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 75 metacercarial cysts of *E. caproni*. The six infected mice, marked with an ear punch, were separated by gender and housed together in two separate plastic mouse cages ($15 \times 27 \times 14$ cm) and fed Mazuri rodent food (PMI Nutrition, Henderson, CO, USA) and water ad libitum. 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 3 weeks PI up to 10 weeks PI.

Sample Preparation

To collect feces, mice were removed from their cages and individually placed in circular plastic containers (15 cm diameter $\times$ 18 cm height) lined with filter paper on the bottom. For each sample, approximately 50 mg of fecal pellets was collected from individual mice after varying periods of isolation. Such collections were made from each of the six exposed and six control mice weekly from 3 to 10 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 of the exposed mice were infected based on the presence of characteristic *E. caproni* eggs.[1] Previous HPTLC work with this model indicated that isolated *E. caproni* eggs did not contribute to the neutral lipid profile of the infected mouse feces.[4] Of the 6 infected hosts, 4 survived through the end of the 10th week PI. Those 4 mice were necropsied at the end of the experiment and yielded 25 ± 10 worms per host.

Lipid Extraction

Feces were homogenized using a 7 mL capacity Wheaton (Millvile, NJ, USA) glass homogenizer. The lipids were extracted in chloroform-methanol (2:1) in a ratio of 20 parts of solvent to one part feces. In order to completely extract the lipids, first the feces were homogenized in approximately 4 mL 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. Then, with a ratio of 4 parts sample volume to 1 part salt solution, Folch et al.[9] 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) in a ratio of 32 μL solvent per 3 mg original mass of feces. 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 500–1000 μL. Samples were used immediately or after storage at −20°C for up to 2 days.

Thin Layer Chromatography

The standard for neutral lipid analysis was the TLC Reference Standard 18-4A (Nu-Check-Prep, Elysian, MN, USA), which contained 100 mg with 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 quantitatively transferred into a 100 mL volumetric flask by washing the original standard container several times with chloroform-methanol (2:1) and emptying the container into the volumetric flask to prepare a standard solution containing 0.200 μg μL^{-1} for each of the components.

HPTLC analysis was performed on silica gel 60 CF_{254} plates (EMD Chemicals, Inc., Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany). These HPTLC plates are 10 × 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 16.0 μL) and reconstituted samples (2.00, 4.00, and 8.00 μL) were applied to the preadsorbent zone in individual lanes with a 10-μL Drummond (Broomall, PA, USA) digital microdispenser.

Plates were developed with the Mangold[10] 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 50 mL of the mobile phase in each trough and a saturation pad (Analtech, Newark, DE, USA) in the trough opposite the one used for plate 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 (PMA) solution, and heated for 10 min at 115°C on a Camag plate heater until blue neutral lipid zones appeared on a yellow background.

In an attempt to quantify methyl esters in the mouse feces, some silica gel plates were developed in the Smith et al.[11] mobile phase, hexane-petroleum ether-ethyl ether-glacial acetic acid (50:20:5:1), because previous work showed that this mobile phase is more effective than that of Mangold et al.[10] mobile phase for separating the fast moving upper neutral lipid zones.

In an attempt to spread out the lower, more slow moving neutral lipid zones and to identify an unknown neutral lipid fraction that migrated between free sterols and free fatty acids from mouse feces previously referred to as X and proposed to be a diacylglycerol in Bandstra et al.,[4] three additional ratios of the Mangold mobile phase (80:10:1, 90:10:1, and 85:15:1) were tested on silica gel plates spotted with a Matreya neutral lipid standard containing diolein, the 18-1A standard, and a feces sample. The plates were developed and neutral lipids were detected with PMA as described above.

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 (0.400–3.20 μg) 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 (μg), and multiplying by 100.

Student's *t*-test was used to determine the significance of data based on the mean ± standard error values of the lipids of a sample population at 3 to 10 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 0.16, 0.22, 0.53, 0.66, and 0.79, respectively, in the Mangold et al.[10] mobile phase. FS, FFA, and TG were identified in the fecal samples based on comigration of their zones with the corresponding standard zones. Similar to the results published in Bandstra et al.[4], Figure 1 shows qualitative differences in several fractions of neutral

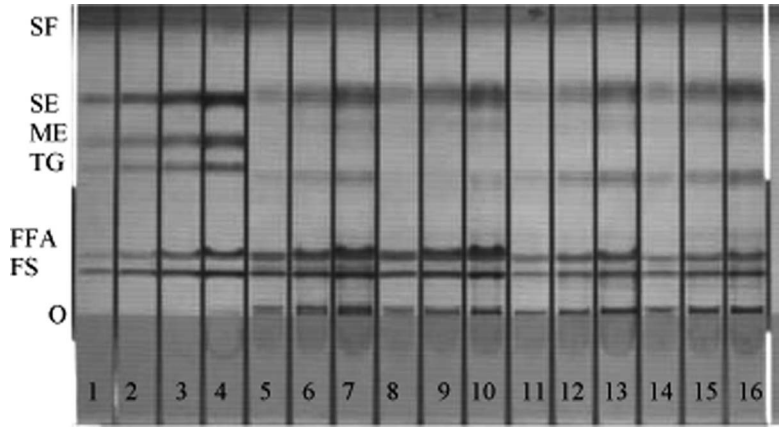

Figure 1. Chromatograms on an EMD Chemicals, Inc. HPTLC plate using the Mangold et al.[10] mobile phase photographed in white light with a Camag VideoStore documentation system showing qualitative increases in free sterols and free fatty acids in 2 samples of feces from infected BALB/c mice (lanes 5–10) versus 2 samples of feces from uninfected BALB/c mice (lanes 11–16) at 7 weeks post-infection. Each sample was applied in 2, 4, and 8 μL aliquots. Lanes 1–4 contain the neutral lipid standard spotted in 2, 4, 8, and 16 μL aliqots, respectively. SF = solvent front, SE = steryl esters, ME = methyl esters, TG = triacylglycerols, FFA = free fatty acids, FS = free sterols, O = origin.

Table 1. Percent by weight (mean ± SE[a]) of neutral lipids in the feces of BALB/c mice infected with *E. caproni* relative to uninfected controls at 3–10 weeks PI

Week PI	Free Sterols		Free fatty acids		Triacylglycerols	
	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected
3	0.6 ± 0.1	0.41 ± 0.06	0.8 ± 0.3	0.41 ± 0.08	0.3 ± 0.1	0.24 ± 0.05
4	1.0 ± 0.1	0.8 ± 0.2	1.0 ± 0.1	0.9 ± 0.2	0.18 ± 0.05	0.4 ± 0.1
5	0.5 ± 0.1	0.39 ± 0.06	0.9 ± 0.2	0.7 ± 0.1	0.18 ± 0.03	0.40 ± 0.06
6	0.7 ± 0.2	0.35 ± 0.07	0.9 ± 0.2	0.52 ± 0.08	0.16 ± 0.03	0.25 ± 0.07
7	0.6 ± 0.2	0.38 ± 0.07	1.0 ± 0.2	0.8 ± 0.2	0.20 ± 0.04[b]	0.46 ± 0.09
8	0.84 ± 0.09	0.68 ± 0.09	0.8 ± 0.3	1.0 ± 0.3	0.18 ± 0.05	0.4 ± 0.1
9	0.56 ± 0.04	0.513 ± 0.003	0.91 ± 0.09	1.02 ± 0.09	0.24 ± 0.01	0.314 ± 0.007
10	0.71 ± 0.04	0.70 ± 0.03	1.0 ± 0.1	1.03 ± 0.03	0.127 ± 0.002	0.34 ± 0.02

[a]SE = standard error.
[b]Value is significantly less than the uninfected value as determined by two-tailed Students *t*-test at the 95% confidence level.

lipids in infected versus uninfected fecal samples utilizing the Mangold et al.[10] mobile phase. Of particular interest in the chromatograms is the apparent increase in the FFA and FS fractions in the feces of infected (lanes 5–10) versus uninfected (lanes 11–16) mice. Data in Table 1 show that while the differences between infected and uninfected feces in these zones were not statistically significant, infected FS zones were considerably more dense at 3 and 5–8 weeks PI, and FFA zones were considerably more dense at 3 and 6 weeks PI, relative to

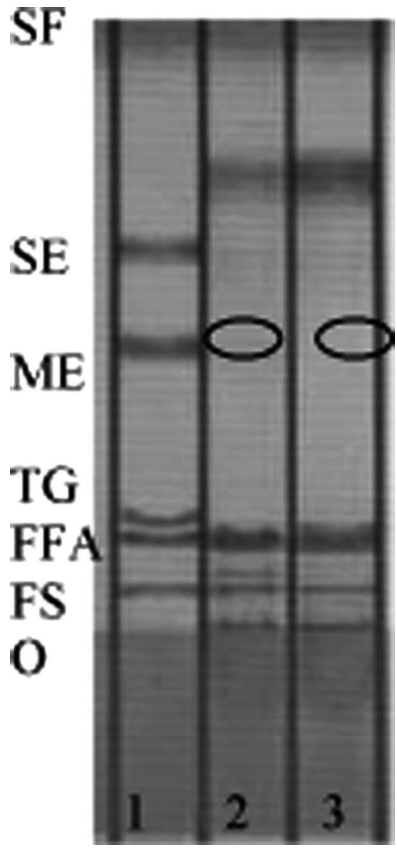

Figure 2. Chromatograms on an EMD Chemicals, Inc. HPTLC plate with the Smith et al.[11] mobile phase photographed in white light with a Camag VideoStore documentation system showing the presence of very light ME zones (outlined with circles) in fecal samples from infected (lane 2) and uninfected (lane 3) BALB/c mice at 7 weeks PI. Each sample was applied in an 8 μL aliquot. Lane 1 contains 16 μL of the neutral lipid standard. See Figure 1 for abbreviations.

the corresponding uninfected zones. The chromatograms in Figure 1 also show a qualitative decrease in the TG fractions of infected feces (lanes 5–10) versus the corresponding fractions of uninfected feces (lanes 11–16). Data from Table 1 show that the TG content of feces from exposed mice was significantly lower at 7 weeks PI relative to those from unexposed mice.

Figure 2 shows that, using the Smith et al.[11] mobile phase, ME were identified as present in the fecal samples based on comigration of its zone with the corresponding standard zone. However, while the ME zones in the feces samples (outlined by circles in lanes 2 and 3) did appear, they were barely visible. Thus, ME were not quantifiable in the samples in any solvent system tested because sample zone scan areas were not bracketed by standard zone scan areas. Additionally, SE were not unequivocally identified in the fecal samples based on their differential migration compared to the CO standard in the Mangold[10] mobile phase. The fast moving ME and SE zones were not further characterized in this study.

Bandstra et al.[4] reported on unknown zones designated X that were considerably less dense in feces from uninfected controls relative to the infected hosts. These zones, with R_F values intermediate between those of FS and FFA, were proposed to be diacylglycerols. However, in all four ratios of the Mangold et al.[10] mobile phase that were tested in this study, standard zones of diolein had R_F values equal to or less than those of FS. This study confirms the fact that the X zones were not diacylglycerols. Additionally, these zones were inconsistent in their appearance and density during the 3–10 week PI period in this study. These results suggest that these zones are not a good marker of *E. caproni* infection in BALB/c mice.

DISCUSSION

Worm infections of about 25 per host induced a significant change in the neutral lipid profiles of the feces of BALB/c mice infected with *E. caproni*. Thus, as first reported in Bandstra et al.,[4] the TG fraction was significantly reduced at 7 weeks PI. Our findings, in accord with those of Bandstra et al.,[4] 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 microscopical fecal examinations to detect echinostome infections in mice. The implication of our findings 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 attempted to obtain an infection level of about 50 worms per host following an initial inoculum of approximately 75 cysts per host, based on a previous study by Balfour et al.[12] on *E. caproni* infection in ICR mice. However, after performing necropsies on all 6 of the infected mice exposed to the 75 cyst burden in this study, it was found that after 10 weeks of infection the BALB/c mice only retained an average of about 25 worms per host. It appears that the ICR mouse allows for a greater worm burden of *E. caproni* than does the BALB/c mouse. Therefore, although we used a greater initial inoculum than did Bandstra et al.,[4] we were not able to get infections with any higher worm burdens than in that study.

Bandstra et al.[4] tentatively referred to an unidentified neutral lipid fraction in the feces of both infected and uninfected hosts as DG based on information in Kates.[13] We have refuted this possibility based on our findings that a diolein standard consistently migrated with R_F values equal or less than those of FS, and the fact that this unknown neutral lipid fraction migrated with an R_F value intermediate between those of FS and FFA. We have no idea of the identity of the unidentified sample zone referred to as X in Bandstra et al.[4]

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