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DETERMINATION OF THE PHOSPHOLIPID AND SPHINGOLIPID CONTENT IN THE FECES OF UNINFECTED BALB/c MICE AND THOSE INFECTED WITH *Echinostoma caproni* BY HIGH PERFORMANCE SILICA GEL THIN LAYER CHROMATOGRAPHY WITH DENSITOMETRY

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SUMMARY

High performance thin layer chromatography (HPTLC) was used to determine the phospholipid and sphingolipid profiles in the feces of BALB/c mice infected with *Echinostoma caproni* (Trematoda) versus uninfected controls. Lipids extracted from fecal samples with chloroform-methanol, 2:1, were separated on silica gel HPTLC plates developed in chloroform-methanol-water, 65:25:4. Densitometric analysis of phosphatidylcholine, phosphatidylethanol-amine, and cerebroside zones was performed to compare infected versus uninfected profiles. No significant changes were found in the phospholipid or sphingolipid profiles in the feces of mice at 1 to 7 weeks post infection compared to matched uninfected controls. Although, our findings suggest that fecal polar lipids are not useful biological markers to distinguish *E. caproni* infections in mice from uninfected controls, the new data presented are important for future comparison in future studies involving feces from mice with heavier levels of infection and urine from infected versus uninfected mice.

INTRODUCTION

Echinostoma caproni infection in mice provides a useful experimental model to study intestinal trematodiasis. It may be possible to generalize information from this model to work on human intestinal trematodiasis [1]. The classical method for the diagnosis of intestinal trematodiasis involves the time consuming and tedious task of examining fecal smears to look for trematode eggs as evidence of adult worm infection. For instance, in studies

on *E. caproni*, the presence of worms in the intestine of the host is determined by the characteristic egg of this trematode [1]. Our laboratory has begun metabolic profiling studies of feces and urine from BALB/c mice infected with *E. caproni* and compared to uninfected controls to determine analytes that may vary as a function of infection. Identification of such analytes would provide the basis of a new diagnostic tool involving the use of high performance thin layer chromatography (HPTLC). HPTLC methods are simple to perform and the results of such studies are relatively easy to interpret.

Previously, our laboratory investigated the neutral lipid content in the feces of mice infected with *E. caproni* versus uninfected mice [2]. We found that compared to the feces from uninfected mice, feces from mice infected with *E. caproni* showed a significant increase in the free sterol fraction and a significant decrease in the triacylglycerol fraction. Albrecht et al. [3] compared the phospholipid and sphingolipid content of the intestinal mucosa of mice infected with *E. caproni* versus uninfected control mice. The major polar lipid fractions detected in the intestinal mucosa of both groups were phosphatidylcholine (PC), phosphatidylethanolamine (PE), cerebrosides, and sulfatides. Because studies of the intestinal mucosa require host necropsy, the Albrecht et al. interventional approach is not useful for metabolic profiling. There are no studies available on the phospholipid and sphingolipid content of feces or urine of mice infected with *E. caproni* versus controls; such information might be useful for the diagnosis of intestinal trematodiasis in animals and in humans. The purpose of this study was to examine the phospholipid and sphingolipid content in the feces of mice infected with *E. caproni* versus uninfected hosts.

EXPERIMENTAL

Mice Maintenance and Infection

BALB/c male mice, each 6 to 8 weeks old and weighing 20-25 g, were used as experimental hosts for *E. caproni* infection. Nine mice were exposed individually by mouth to ca. 50 ± 10 metacercarial cysts of *E. caproni*. The nine infected mice, marked for identification with an ear punch, were housed together in a plastic mouse cage (15 x 27 x 14 cm) and fed Mazuri rodent food (PMI Nutrition, Henderson, CO, USA) and water ad libutum. The nine control mice were handled and treated identically, except that they were not exposed to *E. caproni* metacercarial cysts and 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 7 weeks PI. It is known from a previous study [4] that mice exposed to this number of cysts had ca. 25 ± 10 sexually mature *E. caproni* in the small intestine by 2 weeks PI; also, the infection was long-lived in mice, with worms remaining in the small intestine for a minimum of 2 months PI [4].

Sample Preparation

For collection of feces, mice were removed from their cages and placed individually in plastic circular containers (15 cm diameter x 18 cm high) with filter paper on the bottom. Fecal pellets were collected (ca. 200 mg of feces per sample) after 30 min of isolation. Collections were made from each of the nine infected and six uninfected mice weekly during weeks 1 to 7 PI. Light microscopy of conventional fecal smears on glass slides prepared from the feces of the exposed mice at 2 weeks PI showed that all nine exposed 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 nine infected hosts were necropsied at the end of the experiment and yielded 15 ± 10 worms per host.

Phospholipid and Sphingolipid Extraction

Feces were homogenized in a 7 mL Wheaton (Millville, NJ, USA) glass homogenizer. The polar lipids were extracted in chloroform-methanol, 2:1, in a volume ratio of 20 parts solvent to 1 part feces. Extraction was carried out 2 or 3 times with a total of ca. 7 mL of solvent until the sample was completely homogenized, and the individual extracts were filtered through cotton and collected together in a vial. A solution of 0.88% KCl in deionized water (Folch wash [5]) was added to the extracts in the vial in a volume ratio of 4 parts of sample volume to 1 part salt solution; the combined solutions were vortex mixed, and the top, aqueous layer was removed by pipet and discarded. The bottom layer was evaporated just to dryness under nitrogen gas flow in a water bath (40-60°C) and then reconstituted in chloroform-methanol, 2:1. The reconstitution volume was chosen so that the densitometric scan area of zones in sample chromatograms were bracketed between the lowest and highest scan areas of the standard zones in the calibration graph, and was typically 50.0-200 μL.

HPTLC Analysis

The standard for phospholipid analysis was Polar Lipid Mix no. 1127 (Matreya Inc., Pleasant Gap, PA, USA), containing 25% each of cholesterol,

phosphatidylethanolamine (PE), lecithin (phosphatidylcholine, PC), and lysolecithin; it was dissolved in chloroform-methanol, 2:1, to give a concentration of 0.125 mg mL^{-1} of each component. The standard used for sphingolipid analysis was Matreya Sphingolipid Mix no. 1128, containing equal percentages of cerebrosides, sulfatides, and sphingomyelin; it was dissolved in chloroform-methanol, 2:1, to give a concentration of 0.333 mg mL^{-1} of each component. A phosphatidylserine (PS) standard (Matreya no. 1047) was dissolved in chloroform-methanol, 2:1, at a concentration of 1.00 mg mL^{-1}.

Most aspects of the HPTLC analyses followed the standardized procedures suggested by Reich and Schibli [6]. The plates used contained a 10 x 20 cm silica gel 60 CF_{254} layer with 19 scored lanes and a concentration zone strip at the bottom (Art. No. 13 153; EMD Chemicals, Inc., Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany). Plates were prewashed by development to the upper edge with methylene chloride-methanol, 1:1, in a 20 x 10 cm twin trough chamber (TTC; Camag, Wilmington, NC, USA), dried, and activated for 30 min on a Camag plate heater at 120°C before use. Standards (2.00 to 16.0 µL) and reconstituted samples (1.00 to 8.00 µL) were applied to the concentration zone of individual lanes with a 10 µL digital microdispenser (Drummond, Broomall, PA, USA).

Plates were developed with chloroform-methanol-water, 65:25:4, mobile phase for a distance of 9.0 cm beyond the concentration zone-silica gel interface in a TTC, which required 18-20 min. Mobile phase (25 mL) was placed in each trough and a saturation pad (Analtech, Newark, DE, USA) in the rear trough. After 20 min to allow the chamber to equilibrate, the plate was inserted into the front trough, with the layer facing the saturation pad and the back of the plate resting against the front wall of the TTC.

The developed layer was dried in a fume hood with cool air from a hairdryer for 5 min, sprayed with a 10% solution of cupric sulfate in 8% phosphoric acid, and heated on the plate heater at 140°C for 15 min. The polar lipids were detected as brown-black zones on a white background.

Ninhydrin and α-naphthol spray reagents were also applied to confirm the identity of various zones in sample chromatograms. To detect PE and PS, the plate was sprayed with ninhydrin reagent (0.3 g ninhydrin in 100 mL *n*-butanol and 3 mL glacial acetic acid), air dried for 10 s, resprayed, and heated on the plate heater at 110°C for 10 min [7]. Lipids containing free amino groups appeared as purple-pink spots on a white background. To detect cerebrosides, the plate was sprayed with 0.5% α-naphthol in methanol-water, 1:1, dried at room temperature, sprayed with sulfuric acid-

water, 95:5, and heated at 100°C [8]. Glycolipids, including cerebrosides, appeared as blue-purple spots on a white background.

Automated densitometry of standard and sample zones detected with cupric sulfate reagent was performed with a Camag TLC Scanner II with the following settings: slit width 4, slit length 4, scanning speed 4 mm s^{-1}, and deuterium source 370 nm. The CATS-3 software linear regression program provided a calibration graph relating the standard zone weights to their optimized scan areas. The analyte weights in the sample zones corresponding to their scan areas were automatically determined by interpolation from the graph. Different aliquots of the same sample were usually spotted to assure that at least one scan area was bracketed between the scan areas of the lowest and highest standard; if more than one aliquot was bracketed, the weight interpolated from the calibration graph corresponding to the sample zone area closest to the area of the middle standard was used to calculate the lipid percent.

The percent of lipid was calculated using the following equation:

$$\%w/v = \frac{(IW)(R)(100)}{(SW)}$$

where IW = the interpolated compound weight (µg), R = the ratio of the reconstitution volume (µL) to the volume of sample spotted (µL), and SW = the wet sample weight of feces (µg).

Student's t-test was used to determine significance of data based on the mean percent ± standard error values of the phospholipids and sphingolipids for replicate sample populations at weeks 1 to 7 PI, with $P < 0.05$ being considered significant.

RESULTS AND DISCUSSION

Multiple extractions with chloroform-methanol, 2:1, followed by the use of the Folch procedure gave complete extraction of the lipids from the fecal samples that were clean enough for HPTLC analysis.

It was more convenient to use manual application to the concentration zone with a Drummond digital microdispenser having disposable capillaries rather than an automated Camag Linomat with a 100 µL syringe. Use of the Linomat involves rinsing the syringe with solvent and the next solution to be applied between samples, and the low available volumes of the reconstituted sample solutions in this study would have precluded the sample rinsing step. It has been shown [9] that manual application to a concentration zone plate is as accurate and precise as Linomat application.

Other silica gel HPTLC plates tested were Whatman (Florham Park, NJ, USA) LHPKDF and Analtech HPTLC-HLF, both of which also have 19 scored lanes and a concentration zone. Neither of these plates gave as good resolution of the polar lipids as the EMD Chemicals, Inc. plate for the analyses reported in this paper. The mobile phase used was originally described by Wagner et al. [10] and has been shown in a comprehensive comparative study [11] to be optimum for polar lipid separations on silica gel concentration zone plates.

The detection and quantification limits of the cupric sulfate reagent for the analytes studied were approximately 100 ng. Although the detected zones were colored, it was found that scanning in the ultraviolet mode at 370 nm gave better results than the visible mode of the scanner. Calibration graphs typically had linearity correlation coefficients (*r*-values) of 0.98 for all of the lipids quantified.

The presence of PE, PS, and cerebrosides in sample chromatograms was confirmed by spraying with ninhydrin and α-naphthol reagents. PE and cerebrosides were detected within the detection limits of the densitometer but PS was below the detection limit of the densitometer and consequently not further studied. Sulfatides and sphingomyelin appeared as blue-purple zones in the standard lanes when detected by α-naphthol spray reagent but were not well resolved in the Wagner mobile phase; therefore these compounds were not further examined in this study. The Dragendorff reagent, prepared as described by Kates [8] and purchased premade from Sigma, was used to confirm PC. The use of this reagent was unsuccessful because the entire plate turned the characteristic orange color and no lipid zones were detected, even in standard lanes. Instead, the presence of PC was confirmed by its comigration with the standard. The PC standard also reacted with the ninhydrin spray, as did zones in sample lanes at the same R_F value.

PC, PE, and cerebroside standards had R_F values of 0.33, 0.55, and 0.65, and zones in fecal samples that lined up with these standards and were confirmed as described above were quantified. Table I shows the percent weights of PC, PE and cerebrosides. With respect to phospholipids, visual inspection of the chromatograms showed that the zones for PC and PE appeared identical in the infected and uninfected samples (Fig. 1). This qualitative finding was supported by data from densitometric quantification. In the Wagner solvent system, the cerebrosides in the standard resolved into two very close zones (Fig. 2). Those zones were combined in densitometric analysis. As with the phospholipids examined, visual inspection of

Table I

Percent weight (mean ± standard error) of polar lipids in feces of BALB/c mice infected with *E. caproni* at weeks 1-7 PI

Week PI	Phosphatidylcholine		Phosphatidylethanolamine		Cerebrosides	
	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected
1	0.0137±0.0041[c]	0.0222±0.0069[c]	0.0141±0.0071[b]	0.00362[a]	n.d.	n.d.
2	0.0183±0.0053[f]	n.d.	0.0324±0.011[c]	n.d.	n.d.	n.d.
3	0.0281±0.011[c]	0.0625±0.029[b]	0.0335±0.0061[b]	0.0315±0.019[b]	0.0339±0.0076[b]	0.0277[a]
4	n.d.	0.0340±0.022[c]	0.0137[a]	0.0122±0.00033[c]	0.0424[a]	0.0389±0.019[b]
5	0.0217±0.0076[e]	0.00797±0.0015[c]	0.0380±0.0089[e]	0.0552±0.0057[c]	0.0366±0.013[c]	0.0360±0.014[c]
6	0.0231±0.0029[b]	0.0262±0.011[b]	0.0298±0.0092[e]	0.0228±0.012[c]	0.0148[a]	0.0339±0.00022[b]
7	0.0173±0.0048[d]	0.0314±0.0099[c]	0.0270±0.0087[e]	0.0409±0.015[c]	0.0557±0.023[d]	0.0759±0.020[c]

n.d. – no data obtained; [a] one sample analyzed ($n = 1$); [b] $n = 2$; [c] $n = 3$; [d] $n = 5$; [e] $n = 6$; [f] $n = 8$

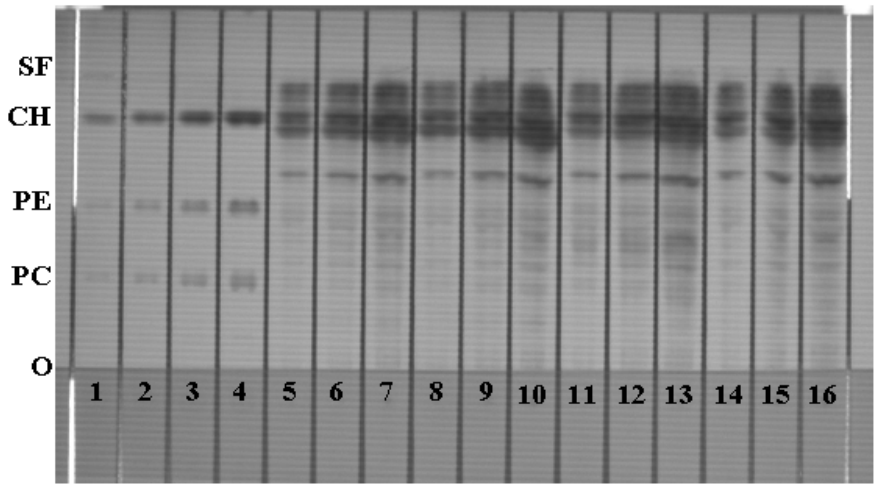

Fig. 1

Chromatograms obtained from an EMD HPTLC plate photographed under white light. Standard lanes were spotted with Phospholipid Mix Standard (lanes 1-4). The two samples from infected mice 3 weeks PI (lanes 5-10) and from the two matched uninfected samples (11-16) do not appear different qualitatively. Quantitative analysis found that the percent weights of these phospholipids in infected versus uninfected samples were not significantly different. Standard lanes were spotted at 2, 4, 8, and 16 µL. Sample lanes were spotted at 2, 4, and 8 µL. SF=solvent front, CH=cholesterol, PE=phosphatidylethanolamine, PC=phosphatidylcholine, O=origin.

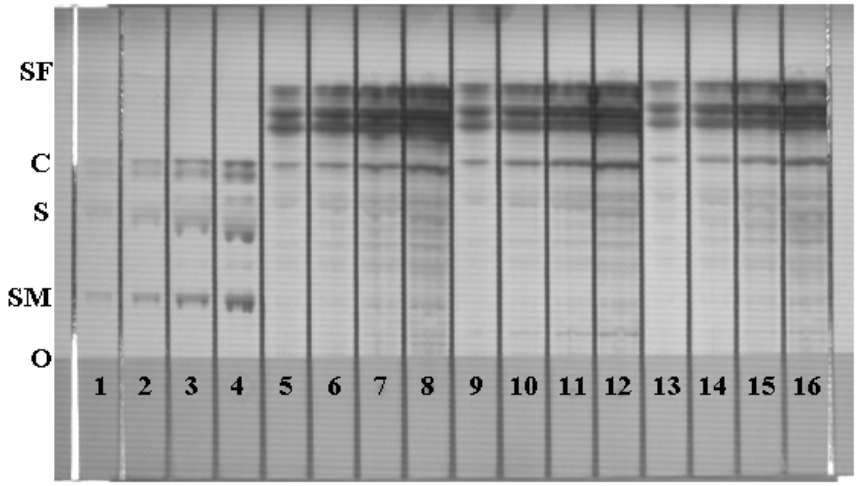

Fig 2.

Chromatograms obtained from an EMD HPTLC plate photographed under white light. Lanes 1-4 contain Sphingolipid Mix Standard spotted at 2, 4, 8, and 16 μL. Infected samples from mice 5 weeks PI (lanes 5-8 and 13-16) versus the uninfected sample (lanes 9-12), spotted at 1, 2, 4, and 8 μL, do not appear to be different qualitatively. Quantitative analysis found no significant differences in the percent weights of cerebrosides in infected versus uninfected samples. SF=solvent front, C=cerebrosides, S=sulfatides, SM=sphingomyelin, O=origin.

the cerebroside zones appeared to be the same in feces from infected versus uninfected hosts. This qualitative finding was also supported by data from densitometric quantification. Our data showed that the analytes examined were not only in low concentrations in mouse feces but there was also great variance in the levels of PC, PE, and cerebrosides in mouse feces, regardless of the infected or uninfected status of the mouse. Albrecht et al. [3] showed a significant decrease in the PC and PE content and an apparent increase in the cerebroside content of the intestinal mucosa of mice infected with *E. caproni* compared to the uninfected controls. Our study on fecal samples showed no significant differences in PC, PE and cerebroside contents in mice infected with *E. caproni* versus uninfected controls. Although Albrecht et al. found significant differences in certain polar lipid compounds in the intestinal mucosa of infected versus uninfected hosts, these compounds were not useful diagnostic markers in fecal samples. The interventional method of Albrecht et al., which involved necropsying the host, makes this method unacceptable for diagnostic purposes. Our metabolic profiling study indicates that the studied phospholipids and

sphingolipids are not useful biological markers of *E. caproni* infection in mouse feces because they do not vary significantly in concentration in infected versus uninfected hosts. Heavier infections of *E. caproni* in mice may significantly affect polar lipid levels in the feces of mice. Future fecal studies will involve mice with heavier infections of *E. caproni* and comparison of urine from infected versus uninfected mice, and the baseline data reported in this paper is important for comparative purposes to these later results.

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