

Sensitive quantitative detection/identification of infectious *Cryptosporidium parvum* oocysts by signature lipid biomarker analysis David. C. White^{1,2}, Srinivas Alugupalli³, David P. Schrum³, Sean T. Kelly³, Monica K. Sikka³, Ronald Fayer⁴ and E. S. Kaneshiro⁵.

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Unique signature lipid biomarkers were found in the acid-fast oocytes of *Cryptosporidium parvum*. This makes possible the rapid detection/identification and potential infectivity directly from drinking water membrane filter retentates. The signature lipid analysis of oocysts showed marked shifts in lipid composition when subjected to environments that render the oocysts non-infectious to neonatal mice. *C. parvum* oocysts' lipids were analyzed after extraction with the modified one-phase Bligh/Dyer solvent extraction. The lipids were fractionated into neutral lipids, "glycolipids" and polarlipids using silicic acid chromatography, and purified into individual lipid classes by preparative thin layer chromatography. The lipids were subjected to sequential mild alkaline, mild acid and strong acid methanolysis. The hydrolytically released lipid components were then identified by gas chromatography/mass spectrometry (GC/MS). The fatty acids consisted of 16:0, 18:0, 18:2w6, and 18:1w9c which are common to fatty acid profiles of microeukaryotes such as fungi, plus 18:1w7c, 20:2 w6, 20:4w6, 24:0, 26:0, 28:0, and the unusual fatty acid 10-OH C18:0. The "signature" fatty acid biomarker, 10-OH 18:0 was confined to a major and a minor "glycolipid" and exists in ester-linkage. The major component containing ester linked 10-OH 18:0 was shown to be phosphatidylethanolamine (PE) by FTIR, phosphate and hydrogen NMR, and FAB-MS. Using negative-ion GC/MS, the limits of detection (s/n = 2) of authentic pentafluorobenzyl-6-OH 18:0 was 0.5 picogram (0.16 femtomole). This corresponds to the 10-OH 18:0 in between 1 to 10 oocysts. Correlations between the lipid composition of oocysts and infectivity to neonatal BALB/c mice showed a marked shift in fatty acid patterns and a loss of 10-OH 18:0 in the PE. Marked differences in the fatty acid patterns were demonstrated between *C. parvum* and *C. muris* in the polar lipids and neutral lipids of the oocysts.

Introduction: A major problem in the water industry is the ability to detect and remove potentially pathogenic microorganisms

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surfaces, rinsed with running water, and then thoroughly dried. Fresh wood shavings for bedding was replaced daily. Water was available *ad libitum*. Milk replacer was fed twice daily. Calves that were less than 2 months old were orally inoculated with 1.5×10^6 oocysts. Feces were collected directly from the rectum into specimen cups 1 to 5 times daily then held at 5°C in a pepsin-HCl solution (1 volume feces:3 volumes pepsin-HCl) during peak oocyst shedding (~5 days). Pepsin-HCl was removed by repeated centrifugation followed by resuspension in deionized water. The aqueous fecal slurry was suspended in sucrose solution and most fecal debris was removed by continuous flow centrifugation. Supernatant containing oocysts were diluted with deionized water and fine particles were removed by continuous flow centrifugation. The relatively clean oocyst pellet was then purified to render it microbe-free by high speed centrifugation over a CsCl gradient and CsCl was removed by repeated centrifugation and resuspension in sterile deionized water.

Extraction and hydrolysis of lipids : Oocysts (10^5 - 10^9) were extracted in a modified single-phase solvent system which included phosphate buffer [10], at room temperature in chloroform/methanol/potassium phosphate buffer (50mM, pH 7.4) at a ratio of 1: 2: 0.8 (by volume) for 3h. Chloroform and nanopure water will be then added to cause a complete phase separation. The organic phase was then collected and the solvent evaporated under nitrogen at 37°C. The total lipid extract was separated into lipid classes on disposable silicic acid columns into neutral lipid, “glycolipid”, and polar lipid fractions [11, 12]. Each fraction or component after further separation by thin layer chromatography was subjected to sequential hydrolysis conditions modified from [13]. Ester-linked components of the neutral lipid, glycolipid, and polar lipid fractions were trans-methylated in anhydrous methanolic mild KOH at 60°C for 30 min. Ester-linked fatty acids were recovered in hexane as methyl esters for GC/MS analysis. The lower layer was made mildly acidic by addition of glacial acetic acid and the heating repeated. The fatty aldehyde dimethyl-acetals formed from the plasmalogens were recovered in hexane from the mild acid extraction for analysis by GC/MS. The more polar components remained in the methanol. The methanol fraction was combined with water, hexane, chloroform, and 2 M HCl, mixed and then held for 12 hours at 100°C. This treatment resulted in the quantitative hydrolysis of the amide linked extractable hydroxy- and non-hydroxy fatty acids, the sphinganine bases from the ceramides, and the alkyl ether lipids which were recovered by a chloroform extraction. The water contains the amino acids and carbohydrates that are components of

Results: Lipid composition of *C. parvum* oocytes: The fatty acid composition of the polar and neutral lipids of purified *C. parvum* oocysts preserved under conditions associated with infectivity for neonatal BALB/c mice and after freezing are given in Table 1. The polar lipid recovered corresponds to about 1.2×10^{-15} moles/oocyst [15].

Detection and characterization of a "signature" lipid: The "glycolipid" fraction of the lipid extract recovered from the silicic acid fractionation in acetone contained three components separable by thin layer chromatography. Two of the components contained the highly unusual fatty acid 10-OH 18:0 in ester linkage. The 10-OH 18:0 showed characteristic mobility on capillary gas chromatography and a fragmentation pattern with a base peak at m/z 217 with major ions at m/z 73, 271, 339, and 371 (of the TMS derivative) and a major component at m/z 508 (chemical ionization with negative ion detection) for the pentafluorobenzyl ester as that of authentic 6-OH 18:0. The major "glycolipid" component was purified by thin layer chromatography and was shown to be ninhydrin positive and phosphomolybdate positive with the chromatographic mobility in two dimensions of authentic PE. This component lipid containing the ester-linked 10-OH 18:0 was shown to be identical to authentic phosphatidyl ethanolamine (PE) by its IR spectra, ^1H NMR, ^{31}P NMR and FAB/MS.

Sensitivity of the detection of the signature lipid: *C. parvum* oocysts maintained under conditions associated with infectivity [16] contained 0.233 pmoles ester-linked 10-OH 18:0 in about 10^3 oocytes. Analysis of authentic 6-OH 18:0 showed linear response with capillary CG/MS with negative ion detection between 0.5 and 500 picograms. At 0.5 picograms (0.17 femtomoles) the signal to noise level was 2:1. This indicates that between 1 and 10 oocysts of infectious *C. parvum* could be detected with the present technology.

Shifts in lipid patterns with infectivity to neonatal BALB/c mice: The data in Table 1 shows that freezing *C. parvum* oocysts at -70°C which renders them non-infectious [16] results in major shifts in polar lipid ester-linked fatty acid patterns (PLFA) (Table 1). There is a greater than three-fold decrease in PLFA (from 3.8 to 0.75 pmoles/ 10^3 oocysts) with a shift in patterns. With loss of infectivity by freezing there is a striking decrease in the proportion of 18:1 ω 9c, a gain in 18:2 ω 6 and 20:3 ω 6 with little change in the proportion of 16:0. There is a gain in the neutral lipid ester-linked fatty acids (increase from 0.99 to 0.74 pmoles/ 10^3 oocysts) with no change in

maintained under conditions where they retain infectivity for BALB/c neonatal mice. Clearly *C. parvum* oocysts must have metabolic activities which are disrupted by freezing at -70°C. Freezing results in a loss of polar lipids and "glycolipid" phospholipids relative to the sterol and neutral lipid fractions as well as shifts in the proportions of the ester-linked fatty acids. It is important to test whether other treatments that render the oocysts non-infectious also show diagnostic shifts in lipid composition. R. Fayer has shown *C. parvum* oocysts are noninfectious after 1, 8 and 24 hours at -70°C [16]. When held at -20°C the infectivity disappeared gradually between 5 and 8 hours and was gone at 24 and 168 hours. At -15°C the cysts remained viable for 24 hours but were non-infectious after 168 hours. Mice infected with oocysts held for up to 168 hours at -5°C or -10°C had developmental stage parasites. The infectivity of a gavage dose of 1.5×10^5 oocysts of *C. parvum* is decreased to between 0 and 25 % after exposure to 72.4°C for 1 minute or 64.5°C for 5 minutes in water [18].

These results suggest strongly that oocysts have metabolic needs to maintain infectivity. Our studies have shown shifts in ratios of triglyceride (TG) relative to other cellular components which have proven a very useful measure of microeukaryote nutritional status [11]. In the green algae *Chlorella* under conditions in which cell division is inhibited, TG accumulates and the polyenoic fatty acid composition of TG and phospholipids decrease [19]. In freezing the oocysts of *C. parvum* the phospholipids decrease and polyenoic PLFA accumulate.

Lipid analysis appears able to provide differentiation between *C. parvum* and *C. muris*. The evidence provided here establishes the high probability that a single direct chemical assay which can be automated provides protection of drinking water systems from *C. parvum* as well as other infectious but non-culturable microbes.

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