LC-MS and LC-MS/MS analysis of bile from mice infected with *Listeria monocytogenes*.

Karolina M. Krasinska[a], Jonathan W. Hardy[b], Lindsay Comeaux[a], Christopher Contag[b], Allis S. Chien[a]
[a]Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University; [b]Department of Pediatrics, Stanford School of Medicine, Stanford, CA

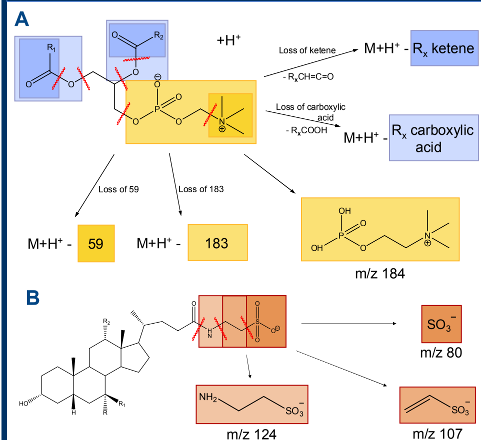
Overview

- Main observed components of mouse bile: phosphatidylcholines (PC), lyso-phosphatidylcholines (lyso-PC) and taurine-conjugated bile acids.
- Highly reproducible LC-MS of bile content allows detection of over 50 species within one LC-MS run and enables comparison across multiple samples.
- Precursor and fragment ion scanning modes are employed in structure elucidation studies.
- Sample dilution proves to be a simple and efficient sample preparation technique.

Introduction

Listeria monocytogenes, a bacterium, causes severe systemic illness, especially in pregnant women. *L. monocytogenes* was engineered for bioluminescence, and sequential images of infection in individual live mice were recorded by detecting the light emission from the bacteria. These studies revealed that *L. monocytogenes* grows in the lumen of the gall bladder (Fig. 5), surviving on pure bile, which has not been described for any other organism in an animal model[1]. We are interested in how the bacteria survive and grow in the pure bile of the gall bladder lumen. To further investigate this phenomenon we have employed LC-MS and LC-MS/MS techniques to study bile samples from infected and uninfected animals, examining fluctuations of bile content at various stages of bacterial infection.

SCHEME 1. Fragmentation pathways for phosphatidylcholines[3] (A) and taurine-conjugated bile acids[2] (B). *See Fig 1. for example spectra.*



Methods

Sample preparation: Female CD1 mice were intravenously infected with bioluminescent *L. monocytogenes*. Bioluminescence images were taken daily to monitor gall bladder colonization (Fig 5). The animals were sacrificed and gall bladder bile samples were extracted and diluted with four volumes of isopropanol. Additional dilution with methanol was necessary prior to LC-MS analysis.

Instrumentation: Analysis was carried out by ESI LC-MS/MS using an Agilent 1100 HPLC and Micromass Quattro Premier triple quadrupole mass spectrometer. HPLC conditions: Atlantis T3 C18 100 x 2.1 mm column, 3 μm particles; gradient 50-99% B in 20 min., hold at 99% for 14 min., total run time 40 min.; A: 2mM ammonium formate in water, B: 1mM ammonium formate in MeOH; 250 μl/min flow rate.

Results

FIGURE 1. MS/MS spectra. Positive ESI spectrum of 16:0/20:4 phosphatidylcholine (A) and negative mode ESI spectrum of taurine-conjugated cholic acid (B) are shown. Characteristic ions at m/z 184 and m/z 80 allow the classification of the species. Other fragment ions provide information about the numbers of carbon atoms and double bonds in the acyl chains of PC molecules.

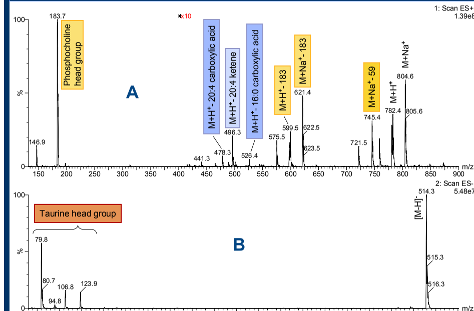
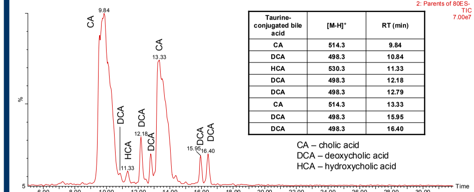


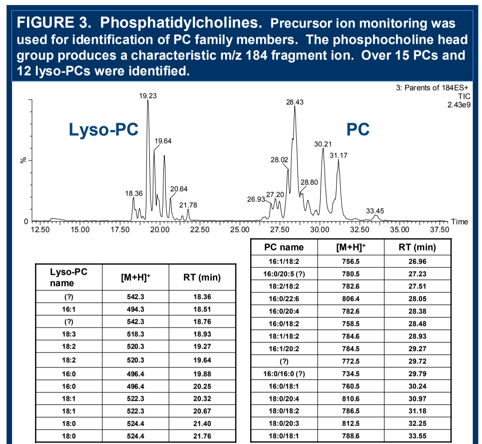
FIGURE 2. Taurine-conjugated bile acids. Precursor ion monitoring in negative scanning mode was used for detection of taurine-conjugated bile acid derivatives. The m/z 79.9 ion represents a fragment of the taurine moiety.



Taurine-conjugated bile acid	[M-H]⁺	RT (min)
CA	514.3	9.84
DCA	498.3	10.84
HCA	530.3	11.33
DCA	498.3	12.18
DCA	498.3	12.79
CA	514.3	13.33
DCA	498.3	15.95
DCA	498.3	16.40

CA – cholic acid
DCA – deoxycholic acid
HCA – hydroxycholic acid

FIGURE 3. Phosphatidylcholines. Precursor ion monitoring was used for identification of PC family members. The phosphocholine head group produces a characteristic m/z 184 fragment ion. Over 15 PCs and 12 lyso-PCs were identified.



Lyso-PC name	[M+H]⁺	RT (min)
(?)	542.3	18.36
16:1	494.3	18.51
(?)	542.3	18.76
18:3	518.3	18.93
18:2	520.3	19.27
18:2	520.3	19.64
16:0	496.4	19.88
16:0	496.4	20.25
18:1	522.3	20.32
18:1	522.3	20.67
18:0	524.4	21.40
18:0	524.4	21.76

PC name	[M+H]⁺	RT (min)
16:1/18:2	756.5	26.96
16:0/20:5 (?)	780.5	27.23
18:2/18:2	782.6	27.51
16:0/22:6	806.4	28.05
16:0/20:4	782.6	28.38
16:0/18:2	758.5	28.48
18:1/18:2	784.6	28.93
16:1/20:2	784.5	29.27
(?)	772.5	29.72
16:0/16:0 (?)	734.5	29.79
16:0/18:1	760.5	30.24
18:0/20:4	810.6	30.97
18:0/18:2	786.5	31.18
18:0/20:3	812.5	32.25
18:0/18:1	788.6	33.55

FIGURE 4. Phosphatidylcholines – in-source fragmentation. Ionization at high cone voltage leads to in-source fragmentation of PC molecules, revealing information about the number of carbon atoms and double bonds in acyl chains. Extracted ion chromatograms are shown: lyso-PC 16:0 and lyso-PC 18:0 give characteristic fragments at m/z 313 and m/z 341, respectively (red). Protonated PCs containing 16:0 alkyl chain lose ketene or carboxylic acid neutral fragments, producing characteristic m/z 496 or m/z 478 ions, respectively (blue).

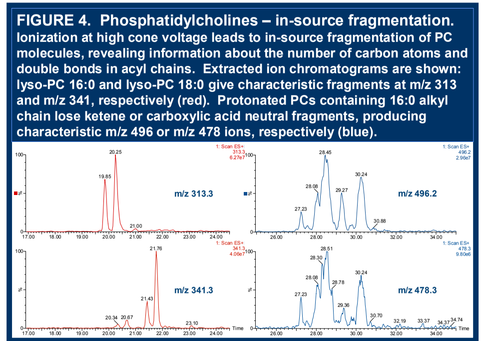


FIGURE 5. *In vivo* bioluminescence imaging – comparative LC-MS. *L. monocytogenes* can replicate extracellularly in the gall bladder. Intravenously infected mice display light emission from the bacteria. Bile samples from uninfected (top) and infected (bottom) animals were analyzed. Chromatograms of full scan MS and m/z 184 precursor ion scanning show significant differences in PC abundances.

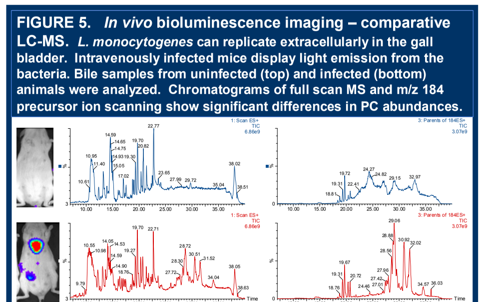


FIGURE 6. MS of Phosphatidylcholines – infected vs. uninfected bile. Precursor ion spectra of m/z 184 show significant differences between uninfected (blue) and infected (red) bile samples. Extracted ion chromatograms of ions of interest demonstrate dramatic changes in abundance.

Conclusions

- Simple sample preparation and LC-MS analysis has been successfully utilized in qualitative analysis of mouse bile, enabling identification of over 30 bile components.
- Several components of bile which are found in uninfected animals are either depleted or non-existent in bile from infected animals.
- These compounds have been identified as phosphatidylcholines, and are suspected to be nutrients on which the bacteria feed when growing in the harsh bile environment.

Future work

- Optimize bacterial growth conditions in *ex vivo* inoculated bile to enable further studies on bile components depleted by the bacteria.
- Continue studies of bile taken directly from infected and uninfected animals in comparison to bile cultures.
- Broaden studies to include porcine and human bile.
- Determine if there are accumulating factors that actively inhibit bacterial growth, such as those sometimes observed with other bacteria.

References

1) Hardy, J.; Francis, K.P.; et al. *Science* **2004**, *303*, 851-853.
2) Kakiyama, G.; Iida, T. et al. *J. of Lipid Res.* **2006**, *47*, 1551-1558.
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Acknowledgements

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