

Executive Summary

Infectious diseases have a substantial global health impact. Clinicians need rapid and accurate diagnoses of infection to direct patient treatment to improve patient outcomes, antimicrobial stewardship, and length of stay. Current methodologies for pathogen detection in the clinical laboratory include biological culture, nucleic acid amplification, ribosomal protein characterization, and genome sequencing. Collectively, these methods are time intensive, require at least 24 hours of incubation of clinically obtained material, and often significantly increase the cost and burden of diagnostic laboratory support. Pathogen identification from single colonies by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of high abundance proteins is gaining popularity in clinical laboratories. Here, we propose a novel and complementary MS approach that utilizes essential, high abundance microbial glycolipids as chemical fingerprints for identification of individual bacterial species. Importantly for clinical use, our glycolipid-based method has three advantages over the protein-based approach: **1)** bacteria can be identified directly from blood culture bottles or urine; **2)** antimicrobial resistant strains can be distinguished from the related susceptible strain in tested cases; and **3)** without the need for culture, the method promises to be more rapid with results possible in one hour or less. Our current mass spectral library has 51 entries of Gram-positive and –negative bacteria, including the so-called ESKAPE pathogens, as well as several fungal species, all which were derived using the same extraction and analysis protocol and the library is expandable.

Description of the Proposed *in vitro* Diagnostic

Approach. Examination of the literature suggests high abundance bacterial glycolipids (see **Figure Appendix I**) could provide species-specific mass spectral profiles due to their immense diversity in the arrangement of fatty acyl side chains and sugar-associated functional groups (1,2). In Gram-negative bacteria, lipopolysaccharide (LPS) comprises the outer leaflet of the outer membrane. The general architecture consists of a lipophilic anchor moiety (lipid A), a core oligosaccharide of arranged hexoses, and an O-polysaccharide chain of repeating subunits (see **Figure Appendix II**). The endotoxin component, lipid A (LA), consists of a glucosamine disaccharide backbone flanked by terminal phosphate residues and fatty acyl chains that extend from the backbone (3). In contrast, Gram-positive bacteria have numerous unique cell wall glycans including cardiolipin and lipoteichoic acid (LTA), which is composed of a diacylglycerol (DAG) lipid that anchors in the membrane and a complex oligosaccharide that penetrates the cell wall (4). We thus hypothesized that these glycolipids represent novel chemical fingerprints that would enable identification of bacteria by MS in a manner similar to bacterial proteins.

Here, we propose a novel diagnostic platform in which microbial membrane glycolipids analyzed by mass spectrometry present chemical “fingerprints” that may then be used to differentiate the ESKAPE pathogens (**Figure 1**), a clinically important subset of bacteria as well as those that exhibit antibiotic resistance by way of chemical modifications to their lipid A structures from their antibiotic sensitive siblings. Furthermore, results are rapid because culturing is not necessary.

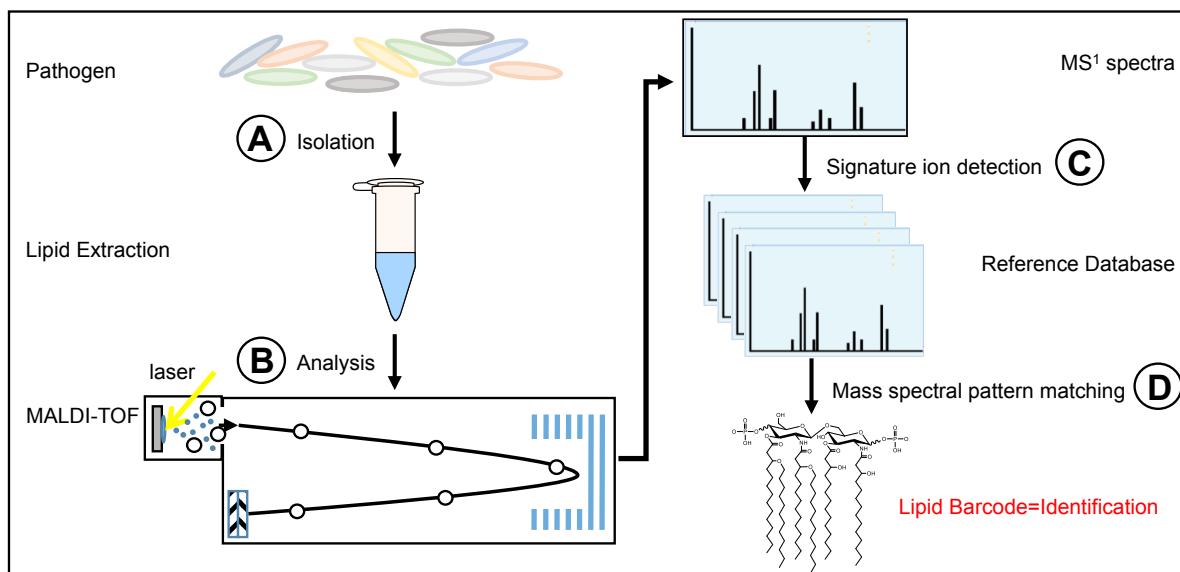


Figure 1. Strategy for glycolipid-based mass spectrometry platform for pathogen identification. **A)** Microbes are isolated from pure culture or biological specimen and whole cell lipids are extracted by hot ammonium-isobutyrate **B)** Lipid extracts are purified and analyzed by MALDI-TOF-MS **C)** A mass spectrum of membrane glycolipids is acquired and compared against an extensive reference database of mass spectral profiles from known organisms via pattern-matching to **D)** Generate a digital identification output and an assigned confidence score.

Using a single optimized protocol, Gram-positive and –negative bacterial glycolipids were extracted from: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. and analyzed by

MALDI-TOF-MS in negative ion mode to obtain glycolipid mass spectra. A library of glycolipid mass spectra was developed using the commercially available Bruker Biotyper platform allowing for identification of the pathogens directly from blood bottles without culture on solid medium and determination of antimicrobial peptide resistance.

To evaluate the diagnostic potential of these membrane glycolipids, we examined the ESKAPE pathogens: Gram-positive *Enterococcus faecium* and *Staphylococcus aureus* and Gram-negative *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., so named for their ability to escape antibiotic treatment. They are of considerable concern due to their prevalence in hospital-acquired infections and acquisition of resistance to antibiotics such as polymyxins (5), a family of cationic antimicrobial peptides (CAMPs) available as polymyxin B sulfate and colistin (polymyxin E). CAMPs bind negatively-charged LPS through electrostatic interactions with the terminal phosphate groups and insert in the bacterial membrane, leading to the permeabilization of the bacterial membrane (6). Emergence of polymyxin resistance has been observed in the ESKAPE pathogens *K. pneumoniae* (7), *A. baumannii* (8), and *P. aeruginosa* (9). When analyzed by MS, we (Figure 2) and others showed that antimicrobial-resistant ESKAPE isolates are chemically distinct from their susceptible counterparts illustrating the potential of our approach to not only identify bacteria but also to improve antibiotic stewardship.

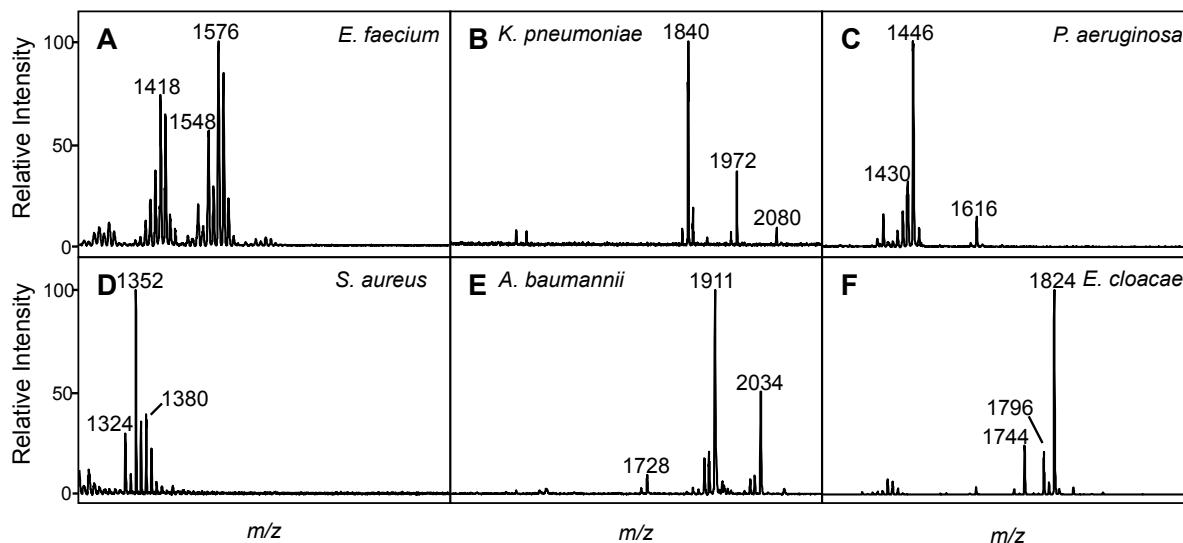


Figure 2. Representative mass spectra from ESKAPE pathogens: A) *Enterococcus faecium*; B) *Klebsiella pneumoniae*; C) *Pseudomonas aeruginosa*; D) *Staphylococcus aureus*; E) *Acinetobacter baumannii*; and, F) *Enterobacter cloacae*. *m/z* values of signature ions are given and were used to develop dot-product analysis in Figure 3.

Our preliminary results demonstrate that bacterial glycolipid mass spectra represent chemical barcodes that identify pathogens, providing a useful, faster (one hour or less) alternative to existing diagnostics allowing the glycolipid library to be expanded to include other pathogens of interest. In this study, we utilize bacterial glycolipid extracts in a manner analogous to the protein-based platform to identify the ESKAPE pathogens by MALDI-TOF-MS. Using a single extraction protocol originally designed for LPS extraction, we were able to differentiate the ESKAPE pathogens by analysis of their mass spectra shown in Figure 2 using dot product analysis the results for which are shown in Figure 3.

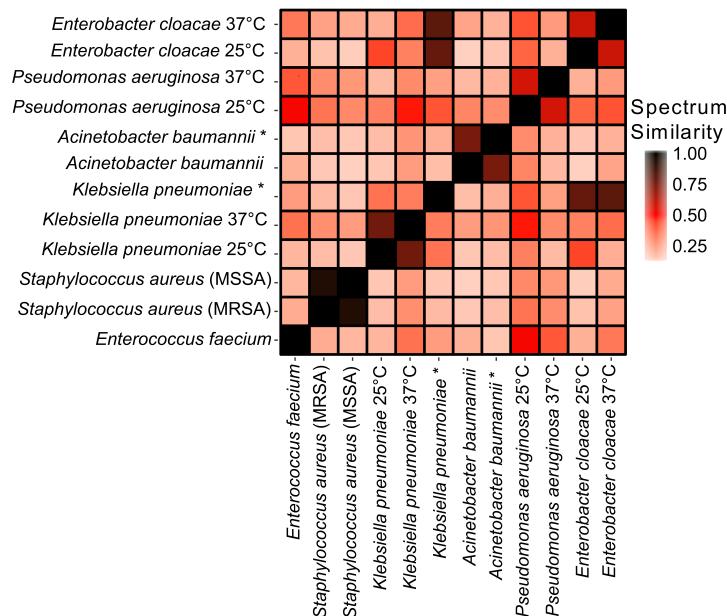


Figure 3. Dot product analysis of mass spectra for differentiation of ESKAPE pathogens. Mass spectra were acquired from lipid extracts of each ESKAPE pathogen. Species were compared by calculating a pairwise dot product between mass lists of ions from each mass spectrum, a measure of spectrum similarity. A similarity score of 1.0 is an identical match (black squares). White squares represent a score of 0.0 where there is no match. (*) indicate colistin-resistant strains.

Our current glycolipid mass spectral library contains 51 unique microbial entries, including the ESKAPE pathogens. This library was built using the same database and software package that is used by the FDA-approved MALDI Biotyper, which is a platform currently used in the clinical laboratory. Importantly for clinical use, the glycolipid-based method has three advantages over the protein-based approach: **1)** bacteria could be identified directly from blood culture bottles (**Figure 4**); **2)** antimicrobial resistant strains could be distinguished from the related susceptible strain in tested cases (**Figure 5**); and **3)** without the need for culture, the method promises to be more rapid (**Figure 4**). These results suggest the potential of developing MS glycolipid profiling as a diagnostic platform that could impact clinical practice.

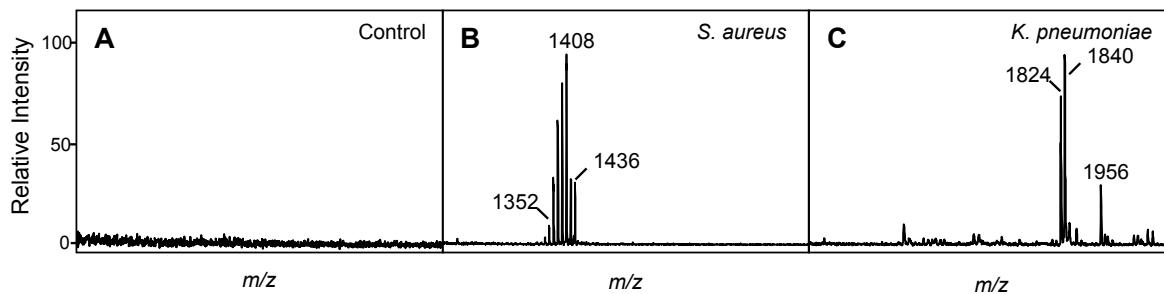


Figure 4. Detection of *S. aureus* and *K. pneumoniae* from blood culture. A) Blood culture control containing sterile blood; **B)** Blood culture containing MRSA M2 after overnight growth (24 hours); and **C)** Blood culture containing *K. pneumoniae* B6 after six hours growth. A 10^4 CFU inoculate was seeded into 10 mL blood, transferred to standard aerobic culture bottles and sampled at 1, 2, 4, 6, and 24 hours. Differential centrifugation allowed separation of human cells, which improves signal/noise in **B**) and **C**) but is not necessary.

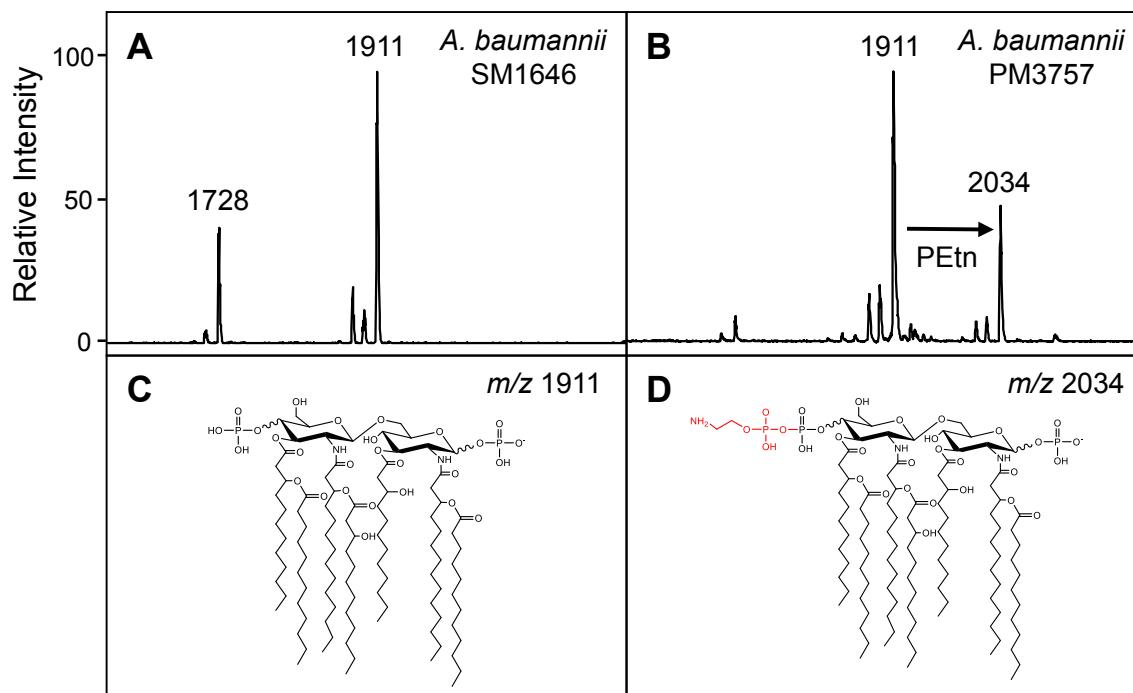


Figure 5. MALDI-TOF-MS of *A. baumannii* with differential colistin resistance. A) Mass spectrum from colistin-susceptible strain TBE1022 with signature ion and **C)** corresponding hepta-acylated LA structure at m/z 1911. Mass spectrum from colistin-resistant strain TBE1027 showing an additional mass peak at m/z 2034 **B)** and corresponding to a phosphoethanolamine (PEtn) addition to one of the terminal phosphates of the base structure that confers resistance **D)**.

Challenges. Our current challenge is primarily related to the way that the existing Biotype software reads our glycolipid mass spectra. The glycolipid mass spectra are much more sparsely populated with ions and have a higher signal to noise ratio than the protein mass spectra for which it was developed. As can be seen in **Figure 3**, dot product analysis correctly distinguishes each organism from the other. However, when the same mass spectra are input to the Biotype software scores vary from 70-98%. We believe this score range can be improved to equal that achieved by the Biotype (i.e. 95-98%) protein scoring routine. The solution lies in the manner in which the glycolipid mass spectra are read by the Biotype software. A preprocessing software tool is being developed to solve this problem. Development of this tool will be the main goal of step 1 funding and is the subject of milestone 3 (*vide infra*). Achieving this milestone will allow our solution to be rapidly adopted by the over 1000 laboratories world-wide that have already adopted the MALDI-TOF-MS hardware.

Another unmet need is to standardize the protocol for sample preparation. This challenge is less significant than the need for software to correctly classify the glycolipid mass spectra. Preliminary results suggest the standard chemical extraction protocol can be optimized to generate mass spectra in less than one hour.

Risks. We are confident that mass spectra for the 51 organisms making up our pilot library contain unique signature ions that identify each organism from all others therein, as shown in **Figure 2** for the ESKAPE pathogens. Literature evidence suggests that many more bacteria and potentially fungi will also have unique signature ions. So the method should be broadly

applicable to Gram-positive and –negative bacteria allowing the library to be expanded as needed by end users. However, it is not clear yet to what extent Gram-positive bacteria will produce unique signature ions that indicate antibiotic resistance as shown by the Gram-negative organisms studied to date which remodel their membranes in response to environmental cues.

While preliminary data suggests that we can produce a standard operating protocol for extraction that results in identifications in under one hour, doing so in a time frame of 10-15 minutes will be challenging. Achieving this very rapid identification may require the use of a different mass spectrometry platform that can carry out multiple reaction monitoring (MRM). This approach is not the subject of this proposal, but may be a viable alternative that can achieve very rapid identifications by focusing on select signature ions rather than collection of entire mass spectra and these instruments are already in wide use in clinical laboratories world-wide.

Finally, we note that the results of a preliminary study are now the subject of a manuscript in review at Proceedings of the National Academy of Sciences (see **Appendix III** for authors, title and abstract).

State-of-the-Art Statement

Infectious diseases pose an ongoing threat to public health. Rapid and accurate pathogen detection is needed to guide physicians in the treatment of infectious diseases to improve patient and economic outcomes (10). In the clinical laboratory, bacteria are routinely identified by morphological and biochemical methods as well as by growth characteristics. Once the identification of the bacteria is known, additional testing such as antibiotic susceptibility testing can be performed to guide definitive antibiotic treatment. Microbiological culture followed by biochemical identification of bacteria is the current gold standard for clinical diagnostics, but this strategy may require additional testing to detect closely related organisms, as with *Enterobacteriaceae* (11). Nucleic acid amplification and sequencing of essential bacterial genes, such as the 16S rDNA, for bacterial identification offers increased accuracy; however, high sensitivity can result in false positives and it may not provide valuable sub-species genotypic information (12). Collectively, these methods are time intensive, require at least 24 hours of incubation of clinically obtained material, and often significantly increase the cost and burden of diagnostic laboratory support. Next generation sequencing is proposed as a culture-free alternative, but it is highly technical with regard to bioinformatic interpretation and is more costly compared to traditional methods (13).

To address some of the current challenges in bacterial identification, mass spectrometric analysis of bacterial proteins is an emerging technology that provides a decrease in turnaround time with an increase in accuracy. Currently, there are two commercially available platforms, the Bruker MALDI Biotyper (14) and bioMérieux VITEK MS (15). These platforms identify bacteria by comparison of a mass spectrum of bacterial proteins from an unknown species to a reference library of previously recorded mass spectra (16). These protein-based platforms have significant limitations, including: requirement for cell culture to obtain pure colonies, poor identification at the species level for closely related species (e.g. *Escherichia coli* vs. *Shigella flexneri*), inability to identify pathogens directly from complex biological samples, and inability to identify antimicrobial resistance, including colistin (polymyxin E) (17). To address the urgent need for novel technologies that can overcome these challenges and expand the ability to rapidly diagnose bacterial infections, we propose the use of highly abundant membrane glycolipids as another class of molecules to exploit for bacterial identification.

Bacterial membranes are composed of lipids of diverse structure and composition (see **Figures in Appendices I and II**). Similar to eukaryotic cell membranes, microbial membranes are composed of a bilayer of amphiphilic glycerophospholipids. In Gram-negative bacteria, there are two distinct membranes separated by a periplasm, whereas in Gram-positive bacteria, the membrane is enclosed by a cell wall (18). Previously, use of bacterial membrane phospholipids had been proposed to phenotype bacteria (11). Specifically, analysis of fatty acids and membrane phospholipids by gas chromatography with flame ionization detection or mass spectrometry (MS) was explored with limited success. Because fatty acid profiles are not unique for each microbial species, species are differentiated via differences in ion intensities, which vary as a function of growth (19-21). This is further complicated by the fact that bacteria and mammalian hosts share some of the same phospholipids; consequently, direct analysis of patient specimens is not possible due to the inability to distinguish bacterial phospholipids from those of the host.

Alternatively, microbial membranes possess more complex glycolipids that are exclusive to bacterial cell membranes and thus are not produced by mammals. They are present in high abundance, approximately 10^6 molecules per bacterium, and are readily extracted from bacteria grown under laboratory conditions or directly from biological fluids. In Gram-negative bacteria, lipopolysaccharide (LPS) comprises the outer leaflet of the outer membrane. The general architecture consists of a lipophilic anchor moiety (lipid A), a core oligosaccharide of arranged hexoses, and an O-polysaccharide chain of repeating subunits. The endotoxin component, lipid A (LA), consists of a glucosamine disaccharide backbone flanked by terminal phosphate residues and fatty acyl chains that extend from the backbone (3). In contrast, Gram-positive bacteria have numerous unique cell wall glycans including cardiolipin and lipoteichoic acid (LTA), which is composed of a diacylglycerol (DAG) lipid that anchors in the membrane and a complex oligosaccharide that penetrates the cell wall (4). Examination of the literature suggests these bacterial glycolipids could provide species-specific mass spectral profiles due to their immense diversity in the arrangement of fatty acyl side chains and sugar-associated functional groups (3,4). We thus hypothesized that these glycolipids represent novel chemical fingerprints that would enable identification of bacteria by MS in a manner similar to bacterial proteins.

Our proposed method described above provides a novel, more rapid, but complementary approach to protein-based bacterial phenotyping that utilizes essential, high abundance microbial glycolipids as chemical fingerprints for identification of individual bacterial species. Importantly for clinical use, our novel glycolipid-based method has three advantages over the protein-based approach: **1)** bacteria can be identified directly from blood culture bottles or urine; **2)** antimicrobial resistant strains can be distinguished from the related susceptible strain in tested cases; and **3)** without the need for culture, the method promises to be more rapid with results possible in one hour or less. Our current mass spectral library has 51 entries of Gram-positive and –negative bacteria, including the so-called ESKAPE pathogens, as well as several fungal species all which were derived using the same extraction and analysis protocol and the library is expandable. Our proposed approach significantly reduces time to identification from the current 24 + hours to less than one by circumventing the need for culture.

Description of Plan to Complete Step 2

Execution. In order to develop a robust platform, we must accomplish the following three milestones.

Milestone 1: Standard operating protocol (SOP) development. Write a Standard Operating procedure for glycolipid extraction and MALDI-TOF MS analysis that is suitable for use by end users in clinical and research laboratories. This is critical because mass spectral libraries must be prepared using the same SOP as test samples. It is thus essential to determine up-front that the SOP produces reproducible data.

Milestone 2: Reproducibility testing and library generation. Use the above developed SOP to demonstrate that the entire procedure from extraction to mass spectral data generation is reproducible.

For each ESACPE pathogen, we will generate data on at least three unique specimens. Each sample will be extracted three times on three different days. From each extraction, five spots will be spotted onto each of two Biotype sample plates, and mass spectra from three different locations on the sample spot will be obtained with a Bruker MS. Thus for each organism we will obtain $3 \cdot 3 \cdot 5 \cdot 2 \cdot 3 = 270$ source mass spectra from which we will examine reproducibility and construct a library.

To evaluate this milestone, we will use the Biotype dendrogram function to generate a dendrogram of all library spectra and all source spectra. Success will be shown if this dendrogram classifies each library mass spectrum with its source spectra, but does not classify any spectra for different microbes together.

Milestone 3: Biotype glycolipid classifier module. Develop a custom classification module for the Biotype classification system, written in MATLAB and a Bruker-proprietary language. Compared to protein fingerprint spectra, glycolipid mass spectra are sparse with higher signal-to-noise. By exploiting these facts the glycolipid Classifier will improve sensitivity and specificity over the standard method developed originally for analysis of bacterial protein mass spectra.

To evaluate this milestone, we will obtain a new specimen for each the ESACPE microbes in the library, prepare replicates as described in Milestone 2, and evaluate mass spectra with the standard Biotype method and the new glycolipid Classifier. Success will be shown if for each ESACPE microbe, the Biotype glycolipid Classifier has at least 90% sensitivity and specificity, and if Biotype glycolipid classifier has better sensitivity and specificity than the standard method.

Data. We will define accuracy and precision of the glycolipid method relative to the existing protein Biotype method by meeting or exceeding the following:

1) *Identify bacteria directly from blood culture bottles in less than 10 minutes.* A recent Biotype study showed that correct identifications were achieved in minutes directly from 76% of 584 positive blood culture bottles after overnight culture on solid medium. It has also been shown that correct identifications were obtained from 80.2% of 212 positive blood culture bottles analyzed.

2) *Have an inter-laboratory reproducibility of at least 98%.* A recent study of Biotype inter-laboratory reproducibility involved 60 samples sent with blind codes to eight laboratories worldwide. Of these 480 samples, 474 were correctly identified in all eight laboratories making inter-laboratory reproducibility ~ 98%.

3) *Produce results as accurate as the Biotype.* The Biotype has an identification accuracy of about 90% versus 80% for traditional biochemical methods. We will achieve a similar accuracy,

but will do so on single colonies and from clinical samples such as blood bottles and urine.

4) *Define failure scenarios.* The Ernst laboratory will generate samples blinded to the Goodlett laboratory. These samples will include Gram-negative and -positive strains. Pathogen identifications from these blinded analyses will be supplied to the Ernst laboratory for decoding. This will allow inter-laboratory reproducibility, e.g. accuracy/precision and sensitivity/specificity, to be estimated.

5) *Define limit of detection (LOD).* The LOD will be defined from both picked pure colonies and from analysis direct from blood bottles and from urine samples.

Detection of New Analyte/Biomarker. Direct detection of antibiotic resistance from specimens like blood bottles and urine is an important aspect of our approach. This direct extraction without culture is viable because mammalian hosts do not make these same classes of glycolipids; see blood bottle control results in **Figure 4A**. For the Gram-negative organisms examined to date that make up the ESKAPE pathogen cohort, we are confident that antibiotic resistance generates unique biomarkers due to chemical modifications of the lipid A structures. We will examine other cases beyond those encompassed by the ESKAPE pathogens such as MSSA versus MRSA to determine if biomarker chemical barcodes exist for these organisms as well.

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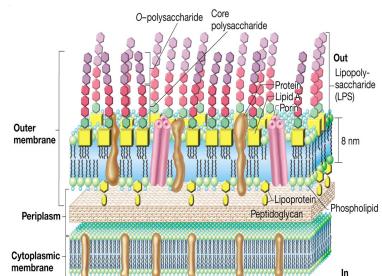
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APPENDIX I

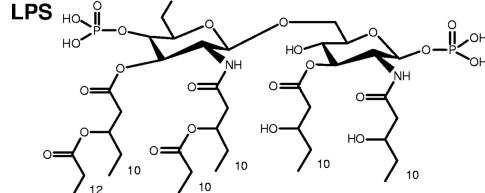
Differences in Microbial and Fungal Membranes

Gram-negative

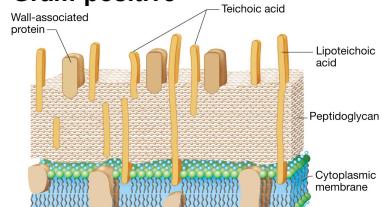


Extractable Membrane Components

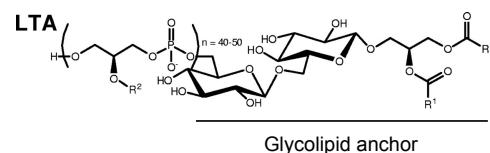
Phospholipids Lipoproteins Lipopolysaccharide



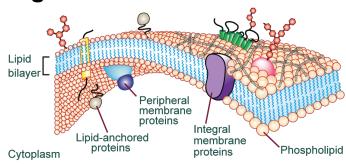
Gram-positive



Phospholipids Lipoteichoic Acid



Fungi



Phospholipids Glycerophospholipids (GP) Sphingolipids (SP) Sterols (S)

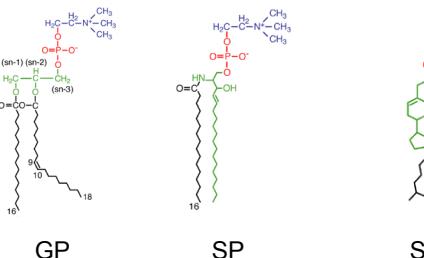


Figure Appendix I. Schematic difference between Gram-positive and –negative bacterial and fungal membrane essential lipids.

APPENDIX II

Differences in Gram-positive and –Negative Glycolipids

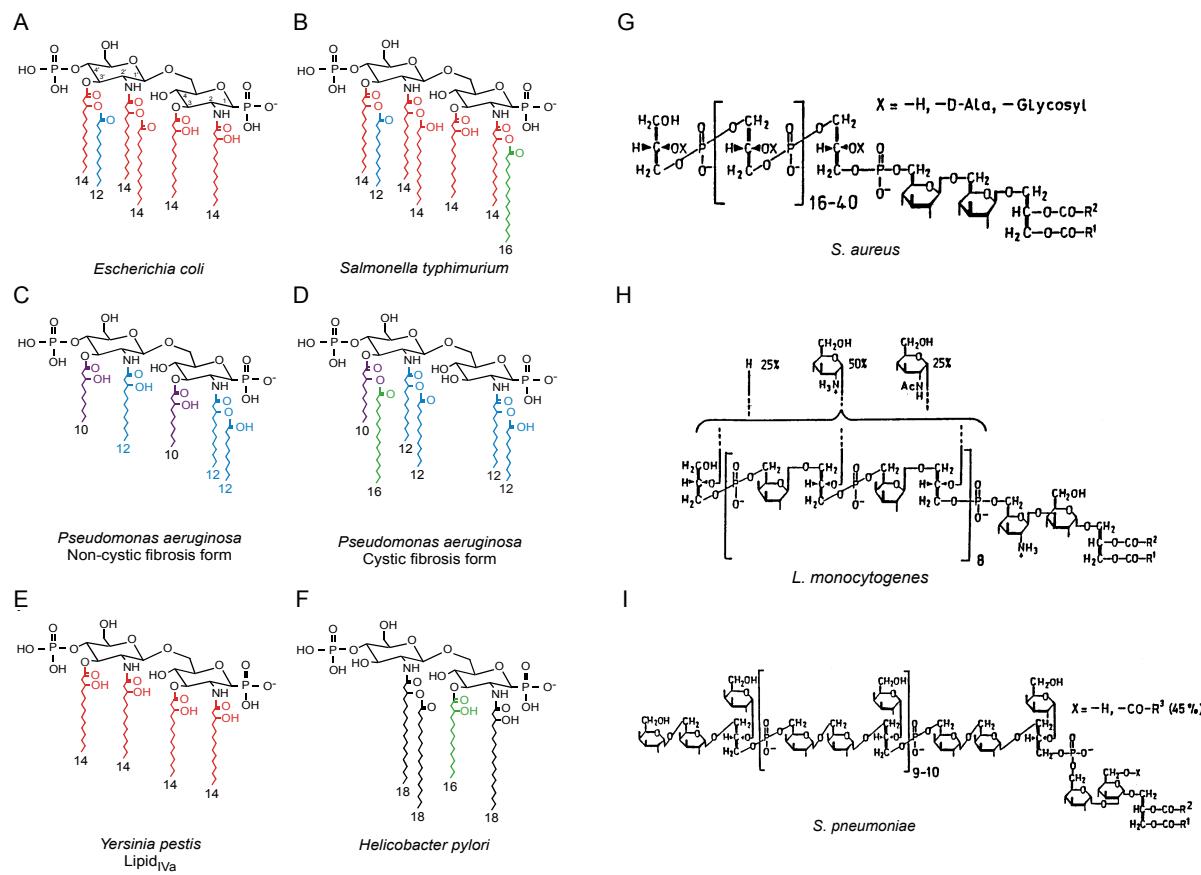


Figure Appendix II. Structural diversity of bacterial lipids. Chemical Structure of LA: (A) *E. coli*, (B) *P. aeruginosa*, (C) *Salmonella typhimurium*, (D) *P. aeruginosa*, (E) *Helicobacter pylori*, and (F) *Yersinia pestis*. Colored structures represent different length fatty acid side chains. Note different structures for *P. aeruginosa* indicating structural diversity depending on location of bacterial isolation. Chemical Structures of LTA: (G) *Staphylococcus aureus*, (H) *Listeria monocytogenes*, & (I) *Streptococcus pneumoniae*.

APPENDIX III

Manuscript in Review at PNAS.

TITLE: Identification of the ESKAPE pathogens by mass spectrometric analysis of microbial membrane glycolipids

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ABSTRACT: Rapid diagnostics that enable identification of infectious agents improve patient outcomes, antimicrobial stewardship, and length of stay. Current methods for pathogen detection in the clinical laboratory include biological culture, nucleic acid amplification, ribosomal protein characterization, and genome sequencing. Pathogen identification from single colonies by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of high abundance proteins is gaining popularity in clinical laboratories. (2) Here, we present a novel and complementary approach that utilizes essential microbial glycolipids as chemical fingerprints for identification of individual bacterial species. Gram-positive and – negative bacterial glycolipids were extracted using a single optimized protocol. Extracts of the clinically significant ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. were analyzed by MALDI-TOF-MS in negative ion mode to obtain glycolipid mass spectra. A library of glycolipid mass spectra was developed using the commercially available Bruker Biolyper platform allowing for identification of the pathogens directly from blood bottles without culture on solid medium and determination of antimicrobial peptide resistance. These results demonstrate that bacterial glycolipid mass spectra represent chemical barcodes that identify pathogens, providing a useful, faster alternative to existing diagnostics.

Public Executive Summary.

Infectious diseases have a significant global health impact. Conventional diagnostics involving microbiological, molecular, and biochemical assays typically require days to weeks to undertake, often yielding inaccurate or incomplete diagnoses. This can have devastating consequences during an infection; therefore, innovative technologies are urgently needed to improve patient outcomes. Mass spectrometry of microbial lipids has been previously explored as a diagnostic through hydrolysis and analysis of the total membrane fatty acids. In our study, we will investigate a novel approach to bacterial identification, namely, analysis of microbial membrane glycolipids (e.g. lipid A) by MALDI-TOF-MS. Prior research has shown that complex glycolipids found in high abundance in bacterial membranes exhibit species-specific structural characteristics that present “signature ions” unique to a given species. We believe that mass spectrometric analysis of these glycolipid fingerprints will allow differentiation and identification of not only the ESKAPE bacterial species shown in this preliminary study but many others and produce practical sub-species information.