APPLICATIONS OF MATRIX ASSISTED LASER DESORPTION/IONIZATION – TIME OF FLIGHT MASS SPECTROMETRY

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MALDI-TOF Microflex applications

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Fundamentals of Matrix-Assisted Laser Desorption/Ionization-Time of Flight MS

- Laser light pulse
- Matrix molecules readily absorb laser light (photons energy), creating an excited energy state
- Localized heating causes micro-explosion of material
- Collisions with residual sample facilitate charge transfer to/from excited matrix molecules

Formation of alternative adducts depends on the presence of respective adduct (either naturally digested or added - depending on type of sample), e.g. - [M+H]+; [M+Na]+; [M+K]+; [M+Cu]+; [M+Li]+; [M+Ag]+.
Following acceleration, the charged ions are allowed to drift through a free field toward the detector.

The speed of travel (time of flight) is proportional to the ion's mass (smaller ions reach the detector first).

MALDI-TOF Mass Analysis – Basic Principle

Linear Mode

Resolution limited due to spatial and energy spreads:
- Spatial spread: initial movement of ions in different directions
- Initial energy (speed) spread: heterogeneous secondary reactions (ion-ion, ion-neutral)
**Resolution,**  
**R=m/Δm**  

Am is a mass peak’s full width at half maximum (FWHM).

Example analysis of a mixture of two compounds X and Y:

- Mass spectrum of single compound X yielding a signal @ m/z X
- Mass spectrum of single compound Y yielding a signal @ m/z Y
- Mass spectrum obtained from mixture [X+Y]: Under these collection conditions, the resolution is insufficient for baseline separation of X and Y. Narrower peaks (i.e. smaller FWHM = higher resolution) would be required for clear separation and accurate labeling.

Δm is a mass peak’s full width at half maximum (FWHM).

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Traditional Workflow: Peptide Mass Fingerprinting (PMF) of Digested Proteins:

- Peak list sent to Mascot (via BioTools)

Appendix B – MALDI matrix structures:

- Sinapinic acid (SA)
- 2,5-Dihydroxybenzoic acid (DHB)
- α-Cyano-4-hydroxycinnamic acid (CCA/CHCA)
- 2,5-Dihydroxyacetophenone (DHAP)
- 3-Hydroxypicolinic acid (HPA)
- 2,4,6-Trihydroxyacetophenone (THAP)
- 2′-(4-Hydroxyphenylazo)benzoic acid (HABA)
- Dithranol (DIT)
- Trans-3-indoleacrylic acid (IAA)
- 1,5-Diaminonaphthalene (DAN)
MALDI Time of Flight MS for Microorganism Identifications

- Figure 1: "Technical description of MALDI-TOF MS. The sample (a single bacterial colony) is mixed with a matrix and spread onto a conductive metal plate. After crystallization of the matrix and microbial material, the metal plate is introduced to the mass spectrometer and is bombarded with brief laser pulses. The desorbed and ionized molecules are accelerated through an electrostatic field and ejected through a metal flight tube subjected to vacuum until they reach a detector, with smaller ions traveling faster than larger ions. Thus, biomolecules separated according to their TOF create a mass spectrum that is composed of mass-to-charge ratio (m/z) peaks with varying intensities. A spectrum is thus a microbial signature that is compared with a database for the identification at the species or genus level."

MALDI Time of Flight MS for Microorganism Identifications

- Bacteria & Yeast – 5,989 isolates
- Mycobacteria – 853 isolates
- Filamentous Fungi – 365 isolates
- Limited number of parasites
- Some viral proteins
- ~ 2,000 species currently
- Ability of laboratory to add to library

MALDI Time of Flight MS for Microorganism Identifications

- Key to rapid identification is good isolation of microorganism on primary plating. 16 to 24 hour old plates.
- Must have single organism for identification to be successful.
- So, still at the mercy of the time it takes to culture the organism.
- Selective media may help as MALDI profile is relatively independent of growth media and reduced chances of mixed cultures.
MALDI Time of Flight MS for Microorganism Identifications

• Validation and calibration
  bacterial test standard applied for each run
• Spread even film of isolated colony in one of the 96 spots on the steel target.
• Apply 1 µl 70% formic acid to lyse the organisms, especially Gram + and capsular organisms. Allow to dry.

MALDI Time of Flight MS for Microorganism Identifications

• Next 1 µl CHCA matrix is applied to each spot and allowed to dry.
• Matrix will facilitate the ionization of the proteins via protonation and an excited energy state.

MALDI Time of Flight MS for Microorganism Identifications

• Vacuum is released to change targets
• Once vacuum recovers, instrument calibration and validation is checked with Bacterial Test Standard (BTS)

BTS = E. coli plus 2 high mass proteins
MALDI Time of Flight MS for Microorganism Identifications

- 240 laser shots are taken of each spot on the template, moving in a pattern to best represent the spot.
- The spectra are automatically smoothed, baseline adjusted then averaged.
- Average spectra is compared to the Biotyper® database for closest matches.

Results indicate the similarity to the organisms in the library.

The identities of the top 10 organisms are reported.

MALDI-TOF Score:

- **2.300…3.000** Highly probable species identification
- **2.000…2.299** Secure genus identification, probable species identification
- **1.700…1.999** Probable genus identification
- **0.000…1.699** Not reliable identification
**Top two IDs are displayed, but clicking on score will show top ten matches.**

**HOW DOES THIS BENEFIT DIAGNOSTIC VETERINARY MICROBIOLOGY?**

1. **Faster diagnosis**—can identify organisms within hours of isolation, developing means for direct detection (no growth) in urine, CSF, serum, or plasma.
2. **More precise identification** of organisms far beyond the reagents and media we could reasonably stock. Can add to the library after 16S rRNA gene sequence confirmation.
3. Ultimately, MALDI-TOF may allow us to identify drug resistant, adherent or toxigenic isolates to speed selection of appropriate therapy.
4. **Reduce costs** of consumables and labor, but hefty capital equipment and maintenance fees.
5. Monitoring new versus recurrent infections, comparing isolates.

**WHAT CAN MALDI-TOF DO FOR RESEARCH?**

1. Reduced costs mean a greater number of cultures may be examined, increasing power especially for epidemiological studies.
2. Ability to build an independent library means incredible specificity, improved characterization, replication, and storage of profiles for future analysis.
3. All types of new opportunities to characterize novel infectious agents: viruses, prions, parasites, plants, algae, etc.
LEPTOSPIRA LIBRARY – PATHOGENIC SEROVARS

- Jennifer Nickolyn-Martin has prepared a library of Leptospira for the MALDI-TOF MS that includes:
  - Leptospira interrogans serovars Autumnalis, Bratislava, Canicola, Icterohemorrhagiae, and Pomona
  - Leptospira kirschneri Grippotyphosa
  - Leptospira borgpetersenii serovar Hardjo.
- Limitation... requires pure culture, so cystocentesis derived urine.

CONSTRUCTING A MASS-SPEC PROFILE (MSP) AND ADDING TO THE LIBRARY

- Tube extraction process:
  - Grow fresh overnight culture in broth or on plates
  - Suspend organism to McFarland 3 in 300 µl water and add EtOH to 70%
  - Pellet and suspend and lyse with 25 µl 70% formic acid. Add an equal volume of acetonitrile.
  - Pellet and spot 1 µl of supernatant on at least 8-10 spots
  - Apply 1 µl of HCA matrix to each spot
  - Shoot each spot 3 times

CONSTRUCTING A MASS-SPEC PROFILE - LIBRARY

- Criteria for generating a new MSP:
  - Include a minimum of 18 - 20 spectra
  - Examine smoothed, baseline adjusted and overlaid spectra; identify outlying peaks
  - Each spectra must have at least 70 peaks
  - Each peak must be present in >70% of the spectra included
  - Remove defective spectra and average to create a library quality MSP.
  - All MSP isolates are confirmed by 16S rRNA gene sequencing
24 STACKED SPECTRA FOR EACH SEROVAR

- Stacked Spectra
  - Zoom to look for missing peaks in high mass range
  - Look for exceptionally high or low amplitude peaks

24 OVERLAIRED SPECTRA FOR EACH SEROVAR

- Overlaid spectra will allow you to examine and remove:
  - Flat lines: low amplitude and missing peaks
  - Outliers: peaks not common to most spectra
  - Spikes: abnormally high amplitude peaks

WITH ZOOM FEATURE, IDENTIFY OUTLIERS

- Excessively high peaks may shift the average of the MSP too high.
- Excessively low peaks may lower the MSP too far.
- Goal is to achieve a uniform average MSP by removing poor spectra and saving 20 that meet minimal criteria
COMPARE EACH SPECTRA TO THE AVERAGE MSP - EACH SHOULD MATCH WITH A SCORE OF ≥ 2.70

PEAK REPORT FOR MSP
- Each spectra must have at least 70 peaks
- Each peak must be present in >70% of the spectra included
- High mass spectra in low quantities may be masked by noise as shown - back to the drawing board
- There is considerable technical skill needed, high reagent quality essential and organism variability so expect a learning curve

DISTANCE BETWEEN 7 SEROVARS - 2-D PLOTS
- 95% scatter plot indicates overlaps of some spectra that will complicate identification.
- When constructing library, focus on conserved spectra that are unique to the targeted serovar
3-D VIEW OF CLUSTERS MAY HELP IDENTIFY OUTLIERS

Once the optimal MSP is generated for each serovar, then you can examine specific protein differences using the gel view (next slide).

TRIPLICATE OR MORE REPRESENTATIVE MSP CAN THEN BE COMPARED USING UNWEIGHTED PAIR GROUP METHOD WITH ARITHMETIC MEAN (UPGMA) - HIERARCHICAL CLUSTER ANALYSIS (HCA)
AVERAGE MSP MAY ALSO BE ALIGNED TO IDENTIFY CONSERVED AND UNIQUE PEAKS

- Multiple isolates from variable sources should be included to increase the diversity of the library in order to identify field variants.
- Most organisms in the current library have 5 or 6 examples, more with highly variable organisms such as E. coli.

WHAT DOES THIS MEAN IN TERMS OF DIAGNOSTIC MICROBIOLOGY?

- Shorter turnaround time – faster diagnoses
- This ability to create specific MSP can be used to identify isolates that differ by as little as one dominant protein.
- This could be the presence or absence of methicillin (penicillin binding protein 2), instantly identifying MRSA or MRSP.
- Unique protein profiles could help trace epidemiology of infections, identify index cases, sources of infection, drift, etc. Recent studies of rhesus and colobus monkeys with Dr. Abigail Wolfe of UIC – DAR.
- We can add new organisms to the database.

Objective 1: Determine the colony-wide prevalence of MRSA carriage

- Multiple Selective media
- Oxacillin sensitivity testing and MIC
- PCR for mecA and blaZ genes
Prevalence Data: Nasal Flora

- **S. aureus**: 50.7%
- **S. aureus + coagulase negative Staph sp.**: 8.0%
- **No Staph isolated**: 11.0%
- **Beta Staph sp.**: 0.7%

82.7% of population has nasal carriage of *S. aureus*

N=300

ALL 300 STAPHYLOCOCCUS AUREUS ISOLATES HAVE BEEN BANKED:

- Minimal inhibitory concentration testing for oxacillin and 21 other antibiotics
- PCR for *mecA* and *blaZ* genes – Dr. Sara Lanka
- MLST performed at U. of Chicago
- MSPs are currently being generated for each isolate for UPGMA analysis using MALDI ClinProTools®

PRELIMINARY DATA SUGGESTS 3 CLADES, BUT WAS ONLY BASED UPON SINGLE SPECTRA, NOT MSP
CANDIDA GLABRATA IN DOLPHINS
- Miranda O'Dell is completing her MS degree in NRES and is examining the role of environmental contamination in dolphin infections with Candida glabrata, a yeast responsible for skin infections and respiratory disease.
- We hope to identify differences between clinical and environmental profiles as well as differences between profiles for 5 participating locations on east versus west coasts and enclosures versus netted housing.
- Proteins will be investigated for their role in pathogenesis during future studies.

WILD TYPE OR VACCINE?
- We are trying to determine if MALDI-TOF can distinguish between Streptococcus equi isolates from wild type strangles infections or isolates due to vaccination related infection.
- We have identified several enzymes that differ based upon Biolog® Microarrays.
- Comparative hybridization shows ~15 SNPs leading to amino acid changes in suspected virulence genes.
- Perhaps differences will appear in the MALDI MS profiles. Faster, less expensive than current sequencing or Biolog® methods to determine infection source.
POTENTIAL FOR MALDI-TOF ASSAY DEVELOPMENT

• Viral protein profiles to look for antigenic drift (mutations) in RNA viruses such as PRRSV and influenza. Potentially an improved typing method.

• Rapid identification of other antibiotic resistant bacteria such as the ESKAPE organisms: Enterococcus, Staphylococcus, Klebsiella, Acinetobacter, Pseudomonas, and Enterobacter.

• Analysis of E. coli and Clostridium perfringens for toxin or fimbriae profiles.

QUESTIONS?

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  • Dr. Sara Lanka
  • Jennifer Nickolyn-Martin
  • Miranda O’Dell
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• Contact us @ 217-333-3577