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Utilization of Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) to Identify Environmental Strains of *Mycobacterium avium* Complex

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Introduction

Species within the *Mycobacterium avium* Complex (MAC) group are found to be both prevalent and persistent in drinking water distribution systems. The MAC is composed of two predominant species: *M. avium* and *M. intracellulare*. These species have the ability to survive drinking water disinfection treatments and to thrive in distribution systems via biofilms. For these reasons, the US Environmental Protection Agency (EPA) has listed MAC on the Contaminant Candidate List (CCL) as a potential contaminant that may require regulation if it is proven to have a significant health burden to the public.

A major challenge associated with environmental MAC isolates is the ability to rapidly identify the isolate to the species level. The tools currently available for identification/speciation can be time-consuming, as well as give ambiguous results due to their inability to clearly differentiate species. The purpose of this study was to evaluate Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) as a means to rapidly speciate MAC environmental isolates.

The research presented will demonstrate the use of MALDI-MS to speciate environmental isolates based on their *m/z* signature. Initially, a database of *m/z* signatures was constructed using MAC reference and type strains. Next, *m/z* signatures from environmental isolates were compared to the database of known *m/z* signatures. Additionally, DNA sequence analyses of genes coded for the heat shock protein 65 (*hsp65*), RNA polymerase subunit B (*rpoB*), and 16S rRNA were used to validate the MALDI-MS determination.

MALDI-MS analysis is ideal for the identification/speciation of environmental isolates. This is primarily due to the minimal sample preparation involved (i.e., the ability to go from culture plate directly to analysis), as well as the short analysis time (<2 min) before a determination can be made. These traits make MALDI-MS a powerful tool suited for environmental monitoring and identification of microbial hazards in drinking water.

Objective

To identify a MAC isolate to the species level by MALDI/MS.

Criteria for Success:

Each species within the MAC must have a unique *m/z* signature that can be used for identification.

Definition: An *m/z* signature is a collection of proteins that are detected by the MALDI-MS that have a specific mass (Da) to charge (ionization state) ratio.

Method

Bacterial Strains

Type strains of MAC were purchased from ATCC and reference strains were provide by Olive View-UCLA Medical Center, Aronson T. *et al.,* 1999.

Membrane Filtration

Mimicking membrane filtration, each reference strain was inoculated into a 500 mL sample volume of sterile DI water. The water was treated with 0.04% cetylpyridinium chloride (CPC) for 30 min, filtered through 0.45 µm pore size membrane filters. The membrane filters were rinsed with buffer and aseptically transferred to Middlebrook 7H10 agar plates containing 500 mg/L of cycloheximide. The filters were examined at weekly intervals for eight weeks.

Sample Preparation and MALDI-MS Analysis

Colonies were picked from the plates and transferred into microcentrifuge tubes containing 5 µL 1% trifluroacetic acid (TFA) in water. A 1 µL suspension of cells was plated onto the MALDI target. One microliter of 40% methanol was added to the cell suspension and allowed to air dry. Once the solvent had evaporated, 1 µl of matrix (sinapinic acid) was added on top of the cells and allowed to air dry before analysis by the MALDI-MS instrument. The sinapinic matrix solution was prepared by dissolving 10 mg in 1 mL of 50% acetonitrile/0.1% TFA.

All mass spectra were acquired using a Biflex III (Bruker Daltonics, Billericia, MA) MALDI-MS with a nitrogen laser (337 nm) operated in positive linear mode. The spectra were the average of four spectra, and each spectrum was the average of 100 laser shots. The summed spectra were smoothed and externally calibrated using the standard calibrant mixture, Protein Calibration Standard I. The [M + H]+ of insulin and myoglobin in this standard were used to externally calibrate the mass spectrometer. The acceleration voltage was maintained at 20 kV, the pulse voltage was maintained at 1300 V, and the extraction delay time was 225 ns. The data files were transferred to XMASS ver. 5.1.5 for automated peak extraction and analysis.

DNA Purification and PCR

DNA was prepared from each of the strains by bead beading the cells for 3 min. DNA was extracted and purified using the DNA-EZ purification kit (GeneRite Inc). Primers for amplification of the *hsp65* and *rpoB* were obtained from Devulder G. *et al.*, 2005 and 16S rRNA primer set from Lane D. *et al.,* 1991.

Data Analysis

MALDI-MS data were binned and converted to presence/absence. Data were analyzed using neighbor-joining as implemented by PAUP ver. (4.0b10) software.

DNA sequence data were edited using BioEdit ver. 7.0.9.0. The resulting sequences were analyzed for homology to known sequences in GenBank using the nucleotide BLAST function (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Raw Data

Strains analyzed by MALDI-MS.

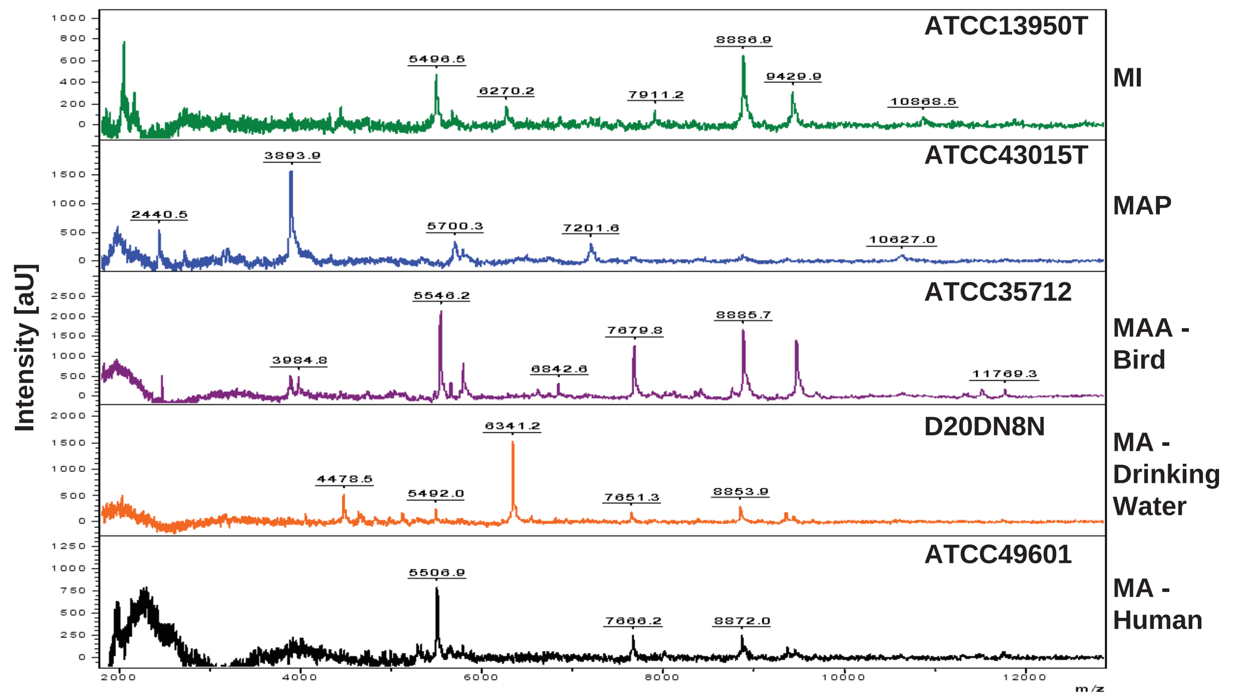


Figure 1.
Representative *m/z* signatures (spectra) of strains in the MAC *M. intracellulare* (MI), *M. avium* subsp. *paratuberculosis* (MAP), *M. avium* subsp. *avium* (MAA), and *M. avium* (MA).

Data Comparison

The raw data were converted to presence/absence. Neighbor-joining analysis was performed using PAUP (4.0b10) to determine strain relatedness.

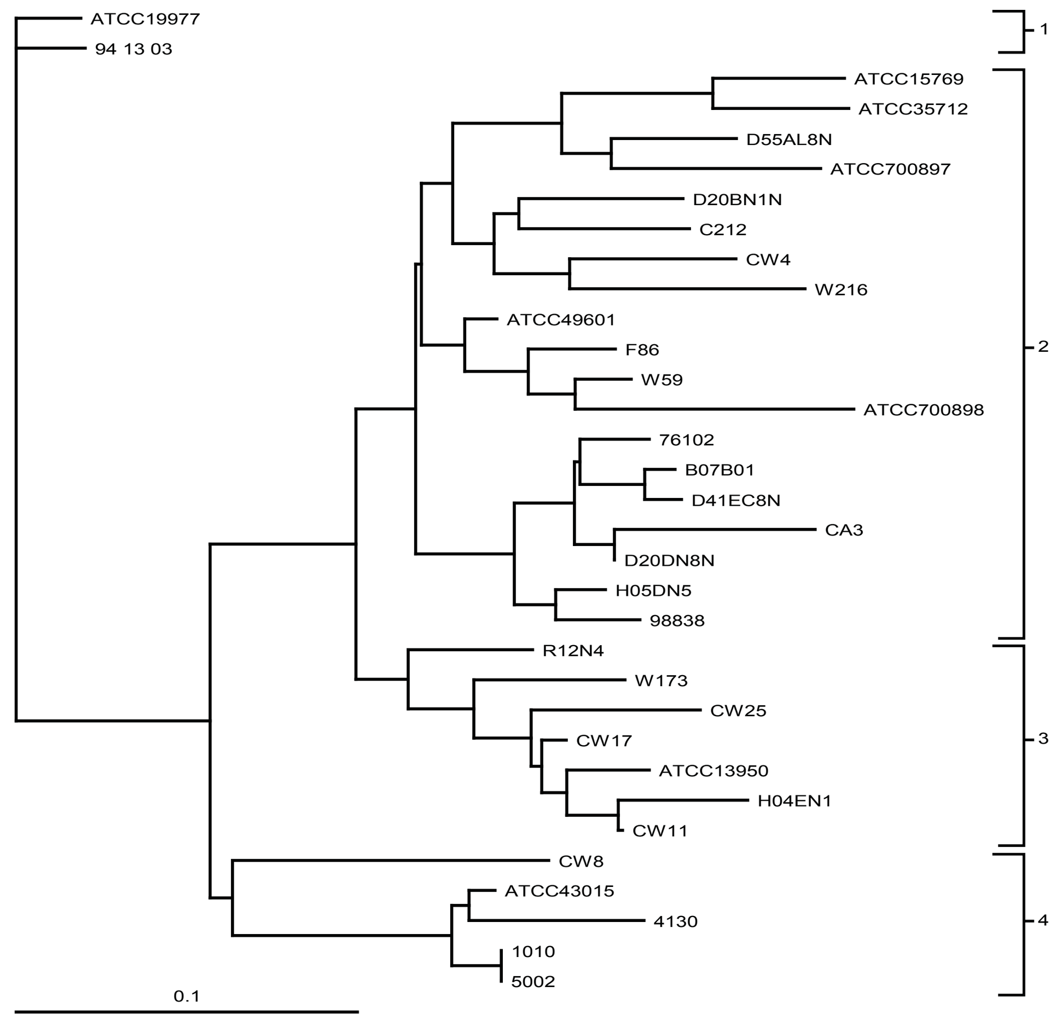
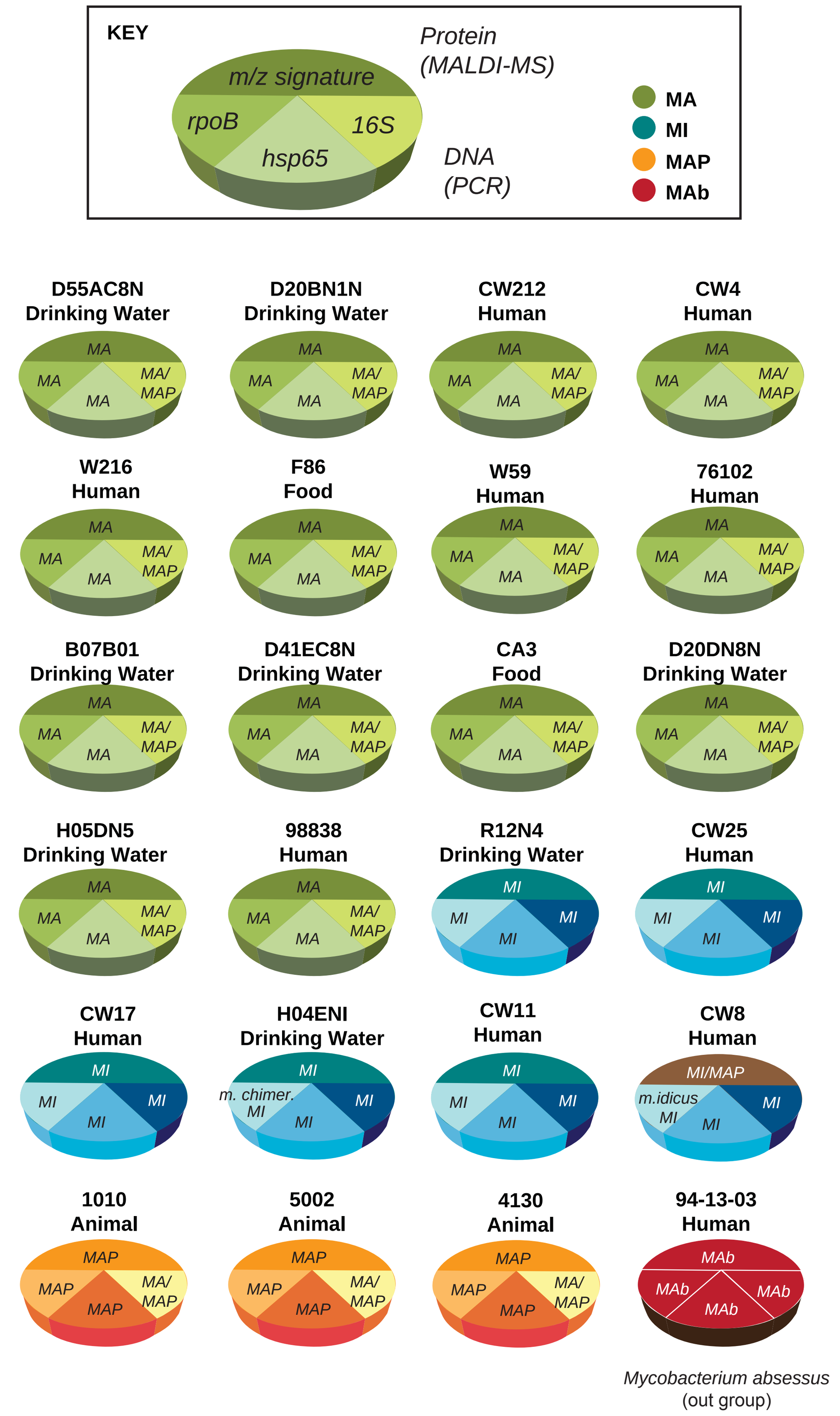


Figure 2.
Neighbor-joining tree generated from the *m/z* signature data.

The strains were clustered into four main groups.
Cluster 1: *Mycobacterium absessus* MAb (out group)
Cluster 2: MA
Cluster 3: MI
Cluster 4: MAP

Validation of MALDI-MS Designation (Species Assignment)



KEY
m/z signature
rpoB
16S
hsp65
Protein (MALDI-MS)
DNA (PCR)
MA
MI
MAP
MAb
D55AC8N Drinking Water
D20BN1N Drinking Water
CW212 Human
CW4 Human
W216 Human
F86 Food
W59 Human
76102 Human
B07B01 Drinking Water
D41EC8N Drinking Water
CA3 Food
D20DN8N Drinking Water
H05DN5 Drinking Water
98838 Human
R12N4 Drinking Water
CW25 Human
CW17 Human
H04ENI Drinking Water
CW11 Human
CW8 Human
1010 Animal
5002 Animal
4130 Animal
94-13-03 Human
Mycobacterium absessus (out group)

Hsp65, rpoB and 16S rRNA genes were sequenced for all strains, excluding the ATCC strains. This information was used to validate the strain designation as determined by the MALDI-MS analysis.

Figure 3.
For all strains tested, the MALDI-MS based species assignment and sequencing information are in AGREEMENT.

Conclusions

Species within the MAC have a unique *m/z* signature that can be used to identify the strains.

Of the species examined, the most diversity was observed in the MA cluster. Three unique *m/z* signatures were observed in this grouping. In the MI cluster, only one *m/z* signature was seen, thus far.

Sequence data and *m/z* data are congruent.

Speciation by MALDI-MS is an ideal technology for any discipline that:

- Generates many cultures (i.e., clinical and environmental).
- Has a need to quickly and accurately speciate a number of unknown isolates (i.e., looking for the needle in the haystack).
- Works with microorganisms that are slow to culture and, as a result, slow to characterize in a timely manner.
- Has a need to rapidly and accurately identify an isolate to species-level.

References

Aronson T. *et al.* 1999. J Clin Microbiol. 37: 1008-1012.

Devulder G. *et al.* 2005. Int J Syst Evol Microbiol. 55: 293-302.

Lane D. 1991. 16S/23S rRNA sequencing, 115-175. In E. Stackebrandt and M. Goodfellow (ed.) Nucleic acid techniques in bacterial systematics. John Wiley & Sons, West Sussex, United Kingdom.