Identification of *Mycobacterium avium* by Direct Matrix-Assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry

Abstract

M. avium is a well known etiological agent for immuno-compromised patients, especially for those with HIV infection. Rapid, accurate discrimination between *M. avium* and other *Mycobacterium* species is essential for appropriate therapeutic management and timely intervention for infection control. Identification and speciation of *M. avium* is both time consuming and labor intensive by traditional bacteriological methods. From our previous report [Yi et al., (1998) Use of Fluorescein Labelled Antibody and Fluorescence Activated Cell Sorter for Rapid Identification of *Mycobacterium species*. Biochem. Biophy. Res. Commu. 250: 403-408.], we have used Flow Cytometry for rapid identification of *Mycobacterium* species. As we mentioned in the text, however, identification of *Mycobacterium* to the species level can be achieved only depending on the availability of species-specific antibody.

In this article, a rapid method involving intact cell mass spectrometry (ICMS) is presented that shows promise for identification, typing and discrimination of *M. avium* from other *Mycobacterium* species. In ICMS, cells from a bacterial colony are emulsified in a chemical matrix, added to a sample probe, dried and analysed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). This technique analyses proteins from intact and disrupted bacteria yielding spectra consisting of 16 peaks from 5000 to 17500, which represent the mass:charge (m:z) ratios. Each peak corresponds to a molecular fragment released from the cell surface during laser desorption. Specimens can be prepared in a few seconds from plate cultures and a spectrum can be obtained within 10 min. Our results show that MALDI-TOF-MS is applicable for rapid identification and differentiation of *M. avium* from, and thus shows its potential for clinical *M. avium* identification, or for epidemiological studies.

KEYWORDS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS); *Mycobacterium avium*; identification

Introduction

Mycobacterium avium is a member of MOTT (Mycobacteria other than *Mycobacterium tuberculosis*) ( ). It is an opportunistic pathogen that can be isolated from soil and water and cause infection and clinical disease in a wide variety of animals and humans (9). *M. avium* is frequently seen in patients who are immunocompromised, such as those with AIDS (5). These infections are usually disseminated and have been shown to contribute significantly to the morbidity of AIDS patients (7). By traditional identification method, it is difficult to differentiate *M. avium* from *M. intracellulare*, and thus they both are grouped to the *Mycobacterium avium* complex (MAC, *M. avium*, *M. intracellulare*, and "nonspecific or X" MAC).

From MAC to MA

Despite the common occurrence of disseminated *M. avium* disease in patients with AIDS, the epidemiology of this infection is incompletely understood. Notably, the predominant source of infection and whether disseminated *M. avium* infection results from reactivation or recent acquisition of infection in human AIDS patients remain unclear ( ). It has recently been demonstrated that water distribution systems may be colonized with *M. avium* (18) and may subsequently serve as a potential source of infection for AIDS patients (43). However, in contrast to these findings, some epidemiologic and clinical studies have failed to find an association between specific environmental sources and human infection (16, 44). These conflicting results may, in particular, illustrate the need for suitable epidemiologic markers for investigation of the sources of *M. avium* infections as well as the routes of transmission, especially because of the large numbers of potential sources for human exposure.

Different laboratory methods, including serotyping (41), multilocus enzyme electrophoresis (45), restriction fragment length polymorphism (RFLP) analysis and hybridization to specific probes (4, 10, 11, 14, 15, 19, 29, 32, 33), and pulsed-field gel electrophoresis (PFGE) (2, 4, 7, 23, 24, 36), have been applied for the purpose of rapid identification and/or typing of *Mycobacterium* species. The last two methods mentioned are DNA-based methods and typically use agarose gel electrophoresis of restriction enzyme-digested genomic DNA which is stained directly with ethidium bromide (PFGE) or which is transferred to membranes and probed with labeled DNA (RFLP analysis). Both techniques are relatively slow and labor-intensive (especially for *M. avium*, whose slow growth can delay the time to retrieval of results), requiring DNA of high integrity and at high concentrations. More recently, PCR-based typing methods have been described (22, 25, 31). The application of PCR to the molecular typing of bacterial species offers the potential for a relatively simple and inexpensive

means of typing bacterial isolates for epidemiologic purposes. One of them, described by Picardeau and Vincent (31), used primers that bound to the ends of insertion elements specific for *M. avium* (I*S1245* and I*S131*1), thus amplifying the DNA between closely spaced copies of these elements.

The potentiality of using MALDI-TOF-MS for the rapid identification or discrimination of intact microorganisms based on the "finger-print" principle was assessed by Claydon et al., (1996). Subsequently, this technique was tested in many bacterial species including identification and specialization of *Haemophilus* (Haag et al., 1998), *Helicobacter* and *Campylobacter* (Winkler et al., 1999), and differentiation between *Staphylococcus aureus* strains (Edwards-Jones et al., 2000).

MALDI is a LIMS method of vaporizing and ionizing large biological molecules including proteins or DNA fragments ( ). The biological molecules are dispersed in a solid matrix (small molecules which strongly absorb laser light) such as nicotinic acid ( ). A UV laser pulse ablates the matrix which carries some of the large molecules into the gas phase in an ionized form so they can be extracted into a mass spectrometer. The mass spectrometric analysis is carried out by determination of the time of flight of the generated molecule ions in a Time-of-Flight Mass Spectrometer. This technique allows precise and rapid molecular weight determination of peptides, proteins, carbohydrates, nucleic acids, and synthetic polymers up to 10^6 Da..

From our previous work, we have reported that fluorescein labelled antibody combined with FACS is a suitable method for rapid identification of clinically significant *Mycobacterium* strains to the genus level, including *M. tuberculosis* and some non-tuberculous *Mycobacterium* (NTM) strains (Yi et al., 1998). There is still some potential drawback in this method, especially in the difficulty of obtaining species-specific antibody for *M. tuberculosis*, *M. avium* and other *Mycobacterium* species, respectively (Yi et al., 1998).

The objective of this study was to evaluate the potentiality of using MALDI-TOF-MS for identifying *M. avium*-specific biomarkers and for rapid differentiation of *M. avium* from other commonly isolated *Mycobacterium* species. Eighty-five (?) MAC isolates from both human immunodeficiency virus (HIV)-positive patients and HIV-negative patients in National Taiwan University Hospital were obtained and initially identified by conventional physiological and biochemical identification method. The isolates were subsequently confirmed by GC-MASS to be MAC strains. Among these strains, *M. avium* strains were further identified by PCR/RFLP of hsp65 gene ( ) and also by hybridization with nucleic

acid probes complementary to the rRNA of the mycobacterial species (AccuProbe Culture Confirmation kits for *M. avium*, and *M. intracellulare*; Gen-Probe, USA). The *M. avium* mass profile was established from these 51 strains identified, and was used for comparison with the profiles obtained from other standard and clinically isolated *Mycobacterium* species strains. Our results show that MALDI-TOF-MS shows its great potential as a powerful tool for rapid identification and typing of *M. avium* strains.

Materials and Methods

***Mycobacterium* strains and culture media.** The standard *Mycobacterium* species strains used in this study including *M. avium* No 31-1, *M. intracellulare*, *M. tuberculosis* H37RV, *M, szulbai* JATA 32-01, *M. scrofulaceum* JATA 31-01, *M. flavescens* JATA 67-01, *M. simiae* KK 44-02, *M. kansasii* KK 11-05, *M. gordonae* JATA 33-01, *M. xenopi* 42-02, *E. coli* JM109, *M. chelonae* JATA 62-01, and *M. fortuitum* JATA 61-01 were obtained from the Department of Laboratory Medicine, National Taiwan University Hospital. Clinical MAC isolates were collected from the National Taiwan University Hospital (Taipei, Taiwan) and Chang-Gun Memorial Hospital (Tao-Yuan, Taiwan) during the period of 1998 to 1999.

Bacterial media were purchased from (Difco, USA).

GC-MASS analysis

Identification of the *M. avium* strain by PCR/RFLP

Each test was performed by following the manufacturer's instructions and with the inclusion of controls.

Sample preparation for MALDI-TOF-MS

After incubation, several colonies were removed using an inoculating loop and placed in a vial containing 30 µl of water: acetonitrile (2 : 1). The entire mixture was vortex mixed for 1 min and then centrifuged at 14 000 rpm for 1 min. The supernatant was removed and added to 30 µl of a saturated sinapinic acid solution. Sinapinic acid (Aldrich, Milwaukee, WI, USA) was dis-solved in water:acetonitrile (2 : 1) until saturation occurred. Samples were spotted on an autosampler plate and allowed to air dry. In the case of samples that were lysed, several colonies of Haemophilus were Ðrst washed with acetonitrileÈwater (2 :1) and then lysed with 10 µl of a 200 mM 0.1% SDS solution. The samples were then desalted by spotting on a 0.25 µm Millipore membrane floating on distilled water. After 30 min the solution was removed and 10 µl were added to 10 µl of the saturated sinapinic acid solution as before. Sample spotting and drying were performed as before.

MALDI⁄TOF-MS analysis

Samples were analyzed on a PerSeptive Biosystems (Framingham, MA, USA) Voyager-DE MALDI/TOF-MS mass spectrometer using 337 nm light from a nitro-gen laser. The mass spectrometer was operated in the positive ion mode and 50 laser shots were averaged in each acquisition. An accelerating voltage of 20 kV was used and a two-point external calibration was per-formed using horse heart myoglobin and sinapinic acid as the calibrants. Two separate cultures of each sample were prepared and each cultured sample was analyzed in triplicate by MALDI/TOF-MS. Reported m/z values can vary by ^0.1%.

were analyzed directly by MALDI-TOF mass spectrometry (MS) from bacterial colonies picked from agar plates.

RESULTS

Isolation and confirmation of the clinically isolated *M. avium* strains

By culture and conventional biochemical tests, a total of 89? clinical MAC isolates were collected during the period of 1998 to 1999 as described in the materials and methods. For further confirmation of the MAC strains, bacterial strains were subcultured onto both Middlebrook 7H11 agar and 7H9 broth media before GC-MASS analysis ( ). Each isolate was tested with the MAC AccuProbe kit (AccuProbe; Gen-Probe, USA) to confirm that the isolates belonged to MAC. Isolates were then tested with the *M. avium*-specific AccuProbe. Isolates that were positive with the MAC-specific AccuProbe but negative with the *M. avium*-specific probe were tested with the *M. intracellulare*-specific AccuProbe. PCR/ digestion of the *hsp65* gene ( ) PCR-restriction enzyme pattern analysis (by BestII and HaeIII digestion) of the *hsp65* gene (Lai et al., unpublished data; 37) were also performed as a supplemented test (data not shown ; or Chou Master thesis?). By using these methods, a total of 56 out of the 89 MAC isolates were identified to be *M. avium*. The

bacterial strains were used for subsequent MALDI-TOF-MASS analysis.

Effect of medium and culture times on the MALDI-TOF-MASS pattern of *M. avium*

To see whether the L-J medium will have effect on the MALDI-TOF-MASS pattern of *M. avium*, L-J medium and standard *M. avium* strains growing on L-J medium were subject to MALDI-TOF-MASS analysis. The peaks that were ranged from m/z 5000 to 20000 were analysed. The results in **Fig. 1A** showed that the pattern obtained from L-J medium is significantly distinct from that from *M. avium* growing on LJ medium. A strong m/z signal at the position of about ***** 1000 is observed clearly in the L-J medium pattern, but is not observed from profiles obtained from *M. avium*, suggesting that L-J medium will not affect the MALDI-TOF-MASS analysis result.

To see whether the time of bacterial culture would have effect on the MALDI-TOF-MASS patterns, *M. avium* (number) was cultured on L-J plates and bacterial colonies were harvested at the time intervals of culture for 14, 21, 28, 35, 42, 49, 56, and 63 days before MALDI-TOF-MASS analysis. The results in **Fig.1B** showed that mass profiles are basically similar among the different sample preparations. This result suggests that the time of culture does not interfere with the *M. avium* mass profile.

The reproducibility of MALDI-TOF-MASS pattern of *M. avium*

To see whether the MALDI-TOF-MASS assay was reproducible, *M. avium* was inoculated onto 3 individual L-J media followed by incubation for 21 days. Bacterial colonies were scratched from the media for the sprectral assay. The results showed that patterns obtained were similar among the three samples **(Fig. 1C)**.

Identify the biomarker for the MALDI-TOF-MASS analysis of *M. avium*

To select for mass spectral peaks as the biomarkers for identification or typing of *M. avium* strains. *M. avium* strains (std strain), A11-1, A16-1, A21, 31-1, 33, 49, 61, and 115 were subject to the MALDI-TOF-MASS analysis, and the peaks appearing common to all three strains were selected. A total of 16 peaks obtained within the range of m/z 5000 to 20000 were selected **(Fig. 2)**. The peaks with increasing m/z ratios were numbered from 1 to 16, as shown in **Table 1**. These biomarkers were used as parameters for comparison and identification of *M. avium* from other *Mycobacterium* species.

Direct identification of *M. avium* strains by MALDI-TOF-MASS

Differentiation of *M. avium* from other *Mycobacterium* species by MALDI-TOF-MASS

The current investigation focuses using *M. avium* strains as our study model. To see whether this technique is applicable for direct identification or typing of *M. avium* from other standard or clinically isolated *Mycobacterium* species, *Mycobacterium* strains of different species are also tested. Bacteria tested included non-pigmented, slow-grower [*M. tuberculosis* (*** and 5 clinically isolated strains); *M. nonchromogenium* (JATA 45-01 and), and *M. intracellulare* (JATA 52-01 and 5 clinically isolated strains)], slow-grower, pigmented under light [*M. simiae* (KK 44-02 and clinically isolated strains) and *M. kansasii* (KK 11-05 and **clinically isolated strains)], slow-grower, pigmented without light [*M. scrofulaceum* (JATA 31-01 and 2 clinically isolated strains), *M. gordonae* (JATA 33-01 and *clinically isolated strains), *M, flavescens* (JATA 67-01 and 2 clinically isolated strains), and *M, szulb(g)ai* (JATA 32-01 and 2 clinically isolated strains)], and 3 rapid-grower [*M. phlei* (KK65-01 and **); *M. fortuitum* (JATA 61-01); *M. chelonae* (JATA 62-01, and **)]. *M. xenopi* (42-02 and *clinically isolated strains).

Bacteria were cultured on the Middlebrook 7H11 agar plate following the normal incubation procedure for each bacterial strain, depending on the growth rate. Colonies were harvested directly from the plate and were directly scratched from the plate for MALDI-TOF-MASS analysis. The m/z profile results were shown in **Fig. 3**, and were summarized in **Table 2**. It was shown from the result that while *M. avium* contains all 16 biomarkers, the other 12 Mycobacterium strains

Discussion:

We report the application of MALDI-TOF-MS for the identification of intact Gram-negative and Gram-positive microorganisms taken directly from culture. Analysis of bacteria from a single colony is possible, allowing the screening of mixed cultures. Sample preparation is simple and the analysis automated, providing spectra within minutes. The spectra obtained allow identification of microorganisms from different genera, different species, and from different strains of the same species. The procedure provides a unique mass spectral fingerprint of the microorganism, produced from desorbed components of the cell wall. Consistent data were obtained from subcultures grown for 3-day and 6-day periods, from the same cultures 1 day later and from fresh subcultures 2 months later.

Research groups at the University of Michigan and Wayne State university demonstrated with MALDI-TOF-MS a clear differentiation between normal and

cancer cells from the MCF-10 breast cell line in the mass range from 10 to 100 kDa.[5] Mass spectra recorded for these two types of cells are shown in Figure 1 below. While MALDI-TOF-MS can be very powerful in solving these identification problems, it suffers from a major difficulty — the appearance of the mass spectrum is frequently strongly dependent on experimental conditions such as laser parameters and composition of matrix and sample solutions. Many researchers have systematically studied the effects of some of these parameters on the quality of the MALDI-TOF-MS results.[6,7]

For those readers who wish to explore more on the applications of MALDI-TOF-MS in various biomedical research problems, references [8-10] is a good start. Finally, an important but yet untouched question is the sensitivity of this technique. In most of the works discussed above, including our own experiments, typically loads some 10^6 to 10^8 cells to the sample probe. This is mainly for routine convenience as this technique is still in the stage of feasibility evaluation and establishing a data bank. If ultimate sensitivity has to be pursued, it has been demonstrated more than once (in favorable cases) that a single cell is sufficient for acquiring useful and definitive mass spectrometric information.[11-12]

This technique was used to not only identify the pathogen, *H. ducreyi*, but also to determine strain differences from different isolates (?).
A biomarker for *M. avium* was centered around m/z 58,268, and *M. avium* was distinguished from other *Mycobacterium* species by its ions at m/z 49,608 and 57,231. *M. avium* could be distinguished from ******by their lack of ions around m/z 58,000 and 61,000 as well as distinguishing biomarkers of lower m/z: 10,074 and 25,478 for C. coli; m/z 10,285 and 12,901 for C. jejuni; m/z 10,726 and 11,289 for C. fetus. MALDI-TOF MS is a rapid and direct method for detection of these potentially pathogenic bacteria from culture.

Gram-negative and Gram-positive microorganisms taken directly from culture. Analysis of bacteria from a single colony is possible, allowing the screening of mixed cultures. Sample preparation is simple and the analysis automated, providing spectra within minutes. The spectra obtained allow identification of microorganisms from different genera, different species, and from different strains of the same species. The procedure provides a unique mass spectral fingerprint of the microorganism, produced from desorbed components of the cell wall. Consistent data were obtained from subcultures grown for 3-day and 6-day periods, from the same cultures 1 day later and from fresh subcultures 2 months later.

because the lengthy subculturing step following initial growth is no longer required. This represents a significant improvement over the normal amount of time required for identiÐcation and speciation of Haemo-philus by the conventional approach (i.e. P48 h from the time that culturing begins).

CONCLUSION

We have shown that MALDI/TOF-MS can be used as an efficient method to identify and determine the species of Haemophilus present in both pure and mixed cultures owing to its ability to detect "ÐngerprintÏ proteins. MALDI/TOF-MS may also be used as a rapid screen-ing technique for Haemophilus because analysis may be performed immediately after growth occurs, thus elimi-nating the necessary additional subculturing needed to perform biochemical studies. This is especially advanta-geous in the diagnosis of chancroid because current testing is both time consuming and laborious. Mass spectrometry will provide inexpensive testing while also allowing the rapid analysis of many samples in short periods of time. We have also demonstrated the use of MALDI/TOF-MS as a rapid method for categorizing di†erent strains of H. ducreyi based on variations in the proÐles of proteins originating from near the surface of the cell. This capability can greatly aid cluster studies of chancroid outbreaks. Future studies will investigate the use of MALDI/TOF-MS for the analysis of clinical samples which would eliminate the need for culture of fastidious organisms.

with H. aphrophilus, as both organisms exhibit very similar biochemical and bacteriological proÐles. Several isolates of H. ducreyi from di†erent patients were also analyzed to determine strain variation which could further establish MALDI/TOF-MS as an epide-miological

tool. Bacterial cultures were re-tested after 1
week of refrigeration and no major di†erences in mass
spectral patterns were found.
Figure 1 illustrates the mass spectral proÐles of the
waterÈacetonitrile-soluble fractions of H. inÑuenzae, H.
aphrophilus, H. parainÑuenzae and H. ducreyi from bac-Figure
1. MALDI/TOF mass spectrum of whole cells from several Haemophilus species: (a) H. influenzae, (b) H. aphrophilus; (c) H. parainfluenzae, (d) H. ducreyi.
(1998 John Wiley & Sons, Ltd. J. Mass Spectrom. **33**, 750È756 (1998)

Figure Legends:

Fig 1 Effect of medium and culture times on the mass spectral profiles of standard *M. avium* strains

Fig 2 MALDI/TOF mass spectrum of whole cells from several *M. avium* strains

Fig 3. Identify the *M. avium* biomarkers based on mass spectral analysis.

Fig 4. Categorization of standard *Mycobacterium* strains based on mass spectral analysis.

Fig. 5. The mass spectral profiles of clinically isolated *M. avium* strains