MALDI-TOF MASS SPECTROMETRY AND ANALYTICAL PROFILING INDEX CHARACTERIZATION OF AIRBORNE STAPHYLOCOCCI

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Indoor air quality is often measured in terms of nonviable contaminants. In the Philippines, the viable components of air have not received as much attention as the nonviable counterparts. Among the viable components of indoor air are airborne bacteria, which are potential health hazards. In this initial study, airborne bacteria present in an indoor cafeteria were characterized. Bacterial isolates from indoor air were analyzed using Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometer (MALDI-TOF MS). Mass spectral analyses of the 64 environmental isolates revealed high proportions of *Staphylococcus* spp., *Bacillus* spp., *Micrococcus* spp., and *Lactobacillus* spp. Among the *Staphylococcus* species identified by MALDI-TOF MS were that of *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus* and *S. lentus*. We further investigated the metabolism profiles of *Staphylococcus* genus using Analytical Profiling Index (API). Notably, MALDI-TOF MS and API provided similar identification of the *Staphylococcus* spp. isolates. To the best of our knowledge, this is the first report of MALDI-TOF MS airborne bacterial identification in the Philippines.

1. INTRODUCTION

Microbial aerosols are air suspensions containing bacteria, fungi, and viruses (Spendlove & Fannin, 1983). Airborne bacteria could be pathogenic and had been linked to nosocomial pneumonia, tuberculosis, and legionellosis (Yang & Heinsohn, 2007). Airborne bacteria could also be opportunistic pathogens and are detrimental to people with compromised immune system.

Recent local efforts reported alarming airborne bacterial counts in indoor air environments as in air-conditioned buses (Austria, Azarcon, Caguioa, & Reyes, 2004) and ship cabins (Pua & Salazar, 2004). Although informative, these studies have failed to identify the airborne bacteria present in indoor air. Indeed, the definitive identification of airborne bacteria is crucial in mitigating airborne-related diseases. In developing countries like the Philippines, the impending threats from airborne bacteria are often discounted due to lack of reliable and

efficient technology that analyzes large number of bacterial isolates.

Identification of bacteria can be executed using biochemical assays, genomics, and proteomics. Biochemical assays involve culture dependent approaches, which are time-consuming and may not give consistent results. The Analytical Profiling Index® (API) is one of the most widely used biochemical assay kit in the market. Genomics is directly related to 16S rRNA sequencing while proteomics is often correlated to mass profiles of the ribosomal proteins. Unlike biochemical assays, genomics and proteomics exploit the conserve nature of RNA and ribosomal proteins, respectively. Currently, genomics and proteomics are used as complementary strategies to detect and specify airborne bacteria (Emerson, Agulto, Liu, & Liu, 2008).

Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) protein profiling has been used in proteomics to identify bacterial isolates (Krishnamurthy & Ross, 1996; Holland, Duffy & Rafii, 1999; Jarman, Daly, Petersen, Saenz, Valentine, & Wahl, 1999). Recently, the use of MALDI-TOF MS to identify clinical and environmental isolates is being recognized (Maier & Kostrzewa, 2007). Relative to biochemical assays and genomics, MALDI-TOF MS identification is cost-effective, time-efficient, and culture-independent. The goal of this study is to assess the reliability and applicability of MALDI-TOF MS in identifying airborne bacteria from local air samples.

2. METHODOLOGY

2.1 Air Sampling

Air samples were collected at the Zaide College Canteen of De La Salle University (DLSU) using a Staplex® MBS- 6 Stage Microbial Air Sampler (Staplex® Air Sampler Division, New York, USA) fitted with culture plates (Figure 1). Five Brain Heart Infusion Agar (BHIA) plates were used with the Staplex® Stage Air Sampler. Sterility tests were conducted prior to air sampling. In addition, PASPORT® Weather/Anemometer Sensor (PASCO Scientific, California, USA) was used to measure temperature, relative humidity, and barometric pressure of the indoor atmosphere (Table 1). Air samples were collected for 30 minutes, every other Wednesdays from January-February 2012. Air samples were collected at 12 noon where the canteen was heavily occupied by students, faculty, and non-academic personnel of DLSU. The five BHIA plates were incubated at 37 °C for 24-48 hours. Bacterial colonies were subsequently picked and re-streaked as needed until pure cultures were isolated. No bacterial colonies were observed in the control experiments (the set-up does not draw air samples from the atmosphere).

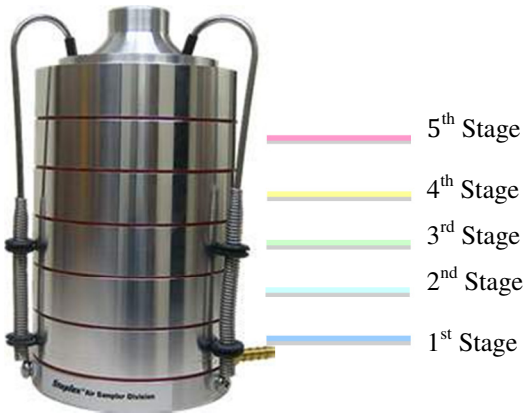

Figure 1. **Staplex® MBS-6 Stages. (Source: www.staplex.com)**

Table 1. ***Atmospheric Conditions in DLSU Zaide College Canteen***

Atmospheric Conditions	Air Samplings		
	1	*2*	*3*
Temperature (°C)	31.3	29.5	29.3
Relative Humidity (%)	44.3	60.1	52.0
Wind speed (m/s)	0.0	0.0	0.0

2.2 Preparation of bacterial sample for MALDI-TOF MS

Fresh growing colonies from the pure culture were dispersed in 300 µL distilled deionized water using sterile disposable inoculation loop

(10 µL, Greiner Bio-one). Absolute ethanol (900 µL) was added and the sample was gently mixed. The resulting cell suspension was centrifuged for 2 minutes at 12,000 rpm. The supernatant was discarded and the washing procedure was repeated. The cell pellet was stored in a freezer and immediately shipped to Bruker Thailand for MALDI-TOF MS analyses.

2.3 MALDI-TOF MS protein profiling

Extraction of proteins was done by the standard formic acid extraction protocol. Briefly, the cell pellet was suspended in 50 µL of 70 % formic acid and the resulting solution was vortexed for one minute. Acetonitrile (50 µL) was mixed into the cell suspension and the mixture was centrifuged for two minutes at 13,000 rpm. The supernatant (1 µL) was transferred into the MALDI target plate and an aliquot of α-cyano-4-hydroxycinnamic acid (HCCA, 1µL) solution was subsequently added. The supernatant-matrix mixture was allowed to air-dry prior to MALDI-TOF MS analyses. The samples were analyzed using a Microflex LT™ instrument and Flexcontrol 3.3 and Biotyper 3.0 database softwares (Bruker Daltonics, Bremen, Germany. Mass spectra within the m/z of 2.0- 20.0 kDa m/z were acquired for the identification of bacteria.

The identification of bacteria by MALDI-TOF MS protein profiling was based on log score. On the basis of peak matches from the database and intensity of correlations, a log score is automatically assigned to the protein profiles (mass spectrum). A score of greater than 1.99 secures species identification while a score of 1.70– 1.99 indicates genus identification. No identification was given if the score was less than 1.70.

2.4 Analytical Profiling Index

The identities of the *Staphylococcus* and *Micrococcus* isolates were determined using Analytical Profiling Index (API). API Staph® (bioMe'rieux, l'Etoile, France) uses 20 biochemical tests for the identification of *Staphylococcus, Micrococcus*, and *Kocuria* spp. Concisely, bacterial inoculum was prepared by suspending young cultures (18-24 hours) into API Staph Medium. The microtubes in the API strip were filled with the bacterial inoculum following manufacturer's instructions. Reading and interpretation of results were executed after 18-24 hours of incubation at 37 °C. The identification of the isolates were revealed using a 7-digit profile number and apiweb™ identification software. The identity of the isolates was assigned and defined using identification percentage (probability of species identification) and typicity index (typical character of the profile studied). Typicity index of greater than 0.25 suggests an acceptable result (Seng, Drancourt, Gouriet, La Scola, Fournier, Rolain, & Raoult, 2009).

3. RESULTS AND DISCUSSION

Ribosomes are the site of protein synthesis in all organisms and are made up of complexes of proteins and RNA. In bacteria, ribosomes are composed of two subunits denoted 50S and 30S. The ribosomal proteins in these subunits are highly conserved between different species of bacteria, are constantly expressed, and are cationic in nature. These features of ribosomal proteins become the basis for the MALDI-TOF MS identification of bacteria. Using the MALDI Biotyper software, one of the isolates was identified as *Staphylococcus epidermidis* (Figure 2).

Figure 2 is a representative example of MALDI-TOF mass spectrum of bacterial isolates. The peaks at m/z 2.0-15.0 kDa represent ribosomal proteins. Twenty to thirty of these ribosomal protein peaks are known while the rest are still unknown. It is noteworthy to mention that four peaks are consistent for *Staphylococcus* genus: 6.6 kDa, 8.1 kDa, 9.3 kDa, and 9.8 kDa. The identities of these proteins were revealed by searching ExPASy database (www.expasy.org). The peaks at 6.6 kDa, 8.0 kDa, and 9.8 represent the L30, L29, and L31 50S ribosomal proteins,

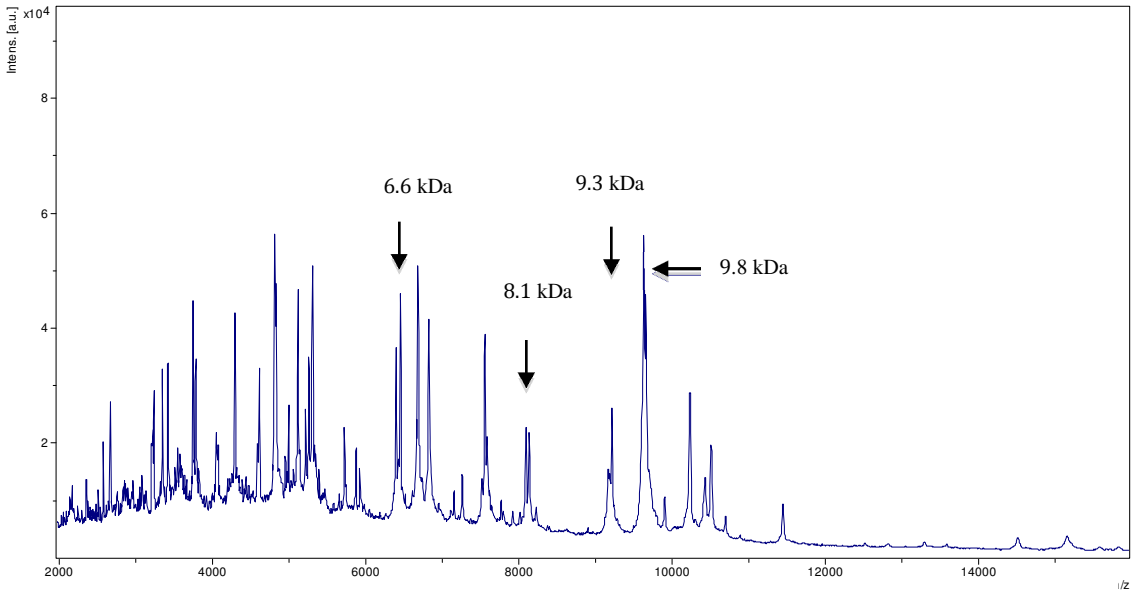

***Figure 2*. MALDI-TOF mass spectrum of *Staphylococcus epidermidis*.**

Table 2. *MALDI-TOF MS characterization of microorganisms in the collected air samples*

Sampling 1	Score	Sampling 2	Score	Sampling 3	Score
Candida krusei	1.830	***Staphylococcus hominis***	**2.097**	***Pseudomonas stutzeri***	**2.151**
Staphylococcus hominis	1.853	***Bacillus cereus***	**2.004**	*Staphylococcus epidermidis*	1.820
Staphylococcus epidermidis	**2.007**	*Brevibacterium casei*	1.994	***Pseudomonas stutzeri***	**2.376**
Bacillus pumilus	1.894	*Micrococcus luteus*	1.935		
Arthrobacter sulfonivorans	1.922				
Staphylococcus epidermidis	**2.118**				
Acinetobacter schindleri	**2.270**				
Aerococcus viridans	1.967	***Staphylococcus hominis***	**2.046**	***Staphylococcus epidermidis***	**2.154**
Lactobacillus hilqardi	1.768	*Lactobacillus delbrueckii*	1.867	*Dermabacter hominis*	1.833
Acinetobacter radioresistens	**2.087**	*Arthrobacter tecti*	1.907	***Staphylococcus lentus***	**2.039**
Brachybacterium faecium	1.879				
Bacteroides suis	1.963	*Aeromonas jandaei*	1.897	***Dermabacter hominis***	**2.201**
Bacilus megaterium	**2.229**	***Staphylococcus hominis***	**2.223**	*Staphylococcus simulans*	1.978
Candida kefyr	1.889	*Bacillus megaterium*	1.869	***Micrococcus luteus***	**2.040**
Staphylococcus epidermidis	1.976	***Staphylococcus saprophyticus***	**2.121**		
Acinetobacter baumannii	1.857	*Micrococcus luteus*	1.785		
Pseudomonas flavescens	1.923	*Arthrobacter castelli*	1.855		
		Rhodococcus ruber	1.791		
Staphylococcus haemolyticus	**2.242**	*Staphylococcus lutrae*	1.894	*Staphylococcus haemolyticus*	1.971
Lactobacillus paraplantarum	1.820	*Micrococcus luteus*	1.709	*Bacillus megaterium*	1.925
Staphylococcus haemolyticus	1.947	*Lactobacillus kimchii*	1.795	***Staphylococcus haemolyticus***	**2.276**
Bacilus alcalophilus	1.819			***Bacillus megaterium***	**2.371**
Clostridium sordelii	**2.003**			*Acinetobacter baumannii*	1.902
Streptomyces avidinii	1.939	*Bacillus pumilus*	1.885	***Staphylococcus epidermidis***	**2.091**
Lactobacilus sakei	1.882	***Micrococcus luteus***	**2.224**	*Bacillus cereus*	1.893
Staphylococcus luqdunensis	1.806	*Staphylococcus sciuri*	1.915		
Staphylococcus epidermidis	**2.188**	*Bacillus cereus*	1.949		
Ochrobactrum tritici	1.820				

Legend: Blue- Stage 1, Turquoise-Stage 2, Green- Stage 3, Yellow-Stage 4, Pink-Stage 5 of Staplex® Air Sampler. Identification up to the species level (log score >2.0) is indicated in bold font.

respectively. In addition, the signal at m/z 9.3 kDa is derived from S18 ribosomal protein of the 30S subunit. The identified ribosomal proteins in Figure 1 have critical functions in apoptosis, DNA repair, and transcription.

By using MALDI-TOF MS protein profiling, 64 isolates have been characterized (Table 2). Forty-one isolates and 23 isolates were identified up to genus and species levels respectively. Identification up to species level (log score >2.0) are indicated in Table 2 in bold fonts. Surprisingly, log scores below 1.8 were not observed. It means that the database of MALDI Biotyper software has the potential to specify environmental isolates. Consolidation of results confers the robustness of MALDI-TOF MS protein profiling in identifying environmental isolates. Interestingly, the results from the three air samplings revealed the rich biodiversity of airborne bacteria at the DLSU cafeteria.

Out of 64 environmental isolates, 32% were *Staphylococcus* spp., 16 % were *Bacillus* spp., 8 % were *Micrococcus* spp., and another 8% were *Lactobacillus* spp. Staphylococci were present in all of the three air samplings. Our data are consistent with previous efforts in Europe and United States where Staphylococci are the most commonly found bacteria in indoor air (Gormy & Dutkiewicz, 2002; Fox, Fox, Elßner, Feigley, & Salzberg, 2010). It is noteworthy that many species of staphylococci are opportunistic pathogens.

Although several publications support the reliability of MALDI-TOF MS for bacterial identification, the potential of this method in the Philippines has yet to be realized. Due to the abundance of staphylococci in the three air samplings, we interrogated the reliability and applicability of MALD-TOF MS based on *Staphylococcus* species. To begin with, we compared the results of MALDI-TOF MS protein profiling to Analytical Profiling Index (API) bacterial identification. The API systems have long been established as the reference technique used to assess the performance of other identification products.

Unlike MALDI-TOF MS, API metabolism profiling uses miniaturized biochemical tests to identify an unknown microorganism. Metabolism profiling has traditionally been used to identify bacteria before the advent of genomics and proteomics.

The figure below represents a metabolism profile of *Staphylococcus epidermidis* using an API Staph® strip (Figure 3). The colored microtubes pertain to the metabolism of sugars (monosaccharides and disaccharides), nitrogen-containing compounds (KNO_3, L-arginine and urea), organic acid (sodium pyruvate), and phosphate-containing compound (β-napthyl phosphate) by *Staphylococcus epidermidis.* During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. For example, the metabolism of mannose can lead to acidification of solution giving a positive yellow color. A positive result depends on the presence of key enzymes that catalyze the catabolism of substrates. Some of these enzymes are often specific for only a number of bacteria. Based on the combination of positive and negative results, a unique numerical profile could be generated for each isolates. The numerical profile is compared with API web database® to yield the identification of the airborne bacteria.

Strikingly, both MALDI-TOF MS and API gave similar identification results for the staphylococcal isolates (Table 3). Congruent results (genus and species) between MALDI-TOF MS and API were considered definite. The results were acceptable because all isolates exhibited typicity indices of 0.5-1.0. Since API Staph® kit can be used to characterized *Staphylococcus*, *Micrococcus* and *Kocuria* spp.; we investigated if the kit could be used to specify *Micrococcus luteus*. The result (Table 3) showed that API Staph® could only identify *Micrococcus luteus* up to the genus level. This incomplete identification is not surprising because API Staph® has low discriminatory power among *Micrococcus* species. To unambiguously identify *Micrococcus luteus* by API, a battery of test must be performed.

***Figure 3*. Metabolism profile of *Staphylococcus epidermidis* using API Staph® strip.**

Table 3. *Comparison of MALDI-TOF MS and API for the identification of Staphylococcus spp*

MALDI-TOF MS Identification	API Identification	API Numerical Profile	API Identification Percentage	API Typicity Index
Staphylococcus epidermidis	*Staphylococcus epidermidis*	6706113	97.9%	1.0
Staphylococcus haemolyticus	*Staphylococcus haemolyticus*	2616151	87.0%	0.64
Staphylococcus hominis	*Staphylococcus hominis*	6626012	74.0%	0.67
Staphylococcus lentus	*Staphylococcus lentus*	6737772	80.1%	0.51
Staphylococcus saprophyticus	*Staphylococcus saprophyticus*	6474153	94.4%	0.63
Micrococcus luteus	*Micrococcus* spp.	0004000	*99.9%*	0.71

These additional tests correspond to increase in consumables and significant increase in workload. The identification results in Table 3 clearly demonstrate the potential of MALDI-TOF MS in analyzing airborne bacteria of local origin and the superiority of MALDI-TOF MS over API.

We analyzed the applicability of MALDI-TOF MS bacterial identification in the Philippine setting. In our experience, MALDI-TOF MS bacterial identification is more cost-effective than API (Table 4).

Table 4. *Comparison of MALDI-TOF MS and API as methods for bacterial identification*

Parameters (per sample)	Methods	
	MALDI	***API***
Preparation and Assay	10 mins	30 mins
Cost	< P 200	~ P 1000
Time to get the identification	< 1 hr	≥18 hrs
Success rate*	> 95 %	~ 50-80%

*According to Boogen, Kostrzewa, & Weller, 2010

For 64 bacterial isolates, the sample analyses for API could take enormous amount of time, space, and resources. Moreover, analyses of different classes of bacteria require different API strips and protocols. For example, API Staph® cannot be used for the analysis of *Bacillus* species and vice-versa. Obviously, API is not the ideal routine method for bacterial identification because of the meticulous assay and the financial constraints associated with it.

MALDI-TOF MS is also more efficient and reliable than API. In terms of fast results, MALDI-TOF MS (< 1 hr) is superior relative to API (≥ 18 hrs). The nature of MALDI-TOF MS as a rapid identification method is crucial especially with the current concerns on approaching pandemics (Tang, Li, Eames, Chan, & Ridgway, 2006). In terms of reliability, MALDI-TOF MS can tolerate minor contaminations without affecting the quality of results (> 95% success rate). On the other hand, these minor contaminations could lead to misidentification of bacteria in API.

Overall, our data suggest that MALDI-TOF MS protein profiling could be used to analyze local airborne microbial flora. The reduced workload and cost also makes MALDI-TOF MS-

based bacterial identification a suitable routine technique for clinical diagnostic laboratories in the Philippines.

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