Instrumental improvements and sample preparations that enable reproducible, reliable acquisition of mass spectra from whole bacterial cells

Pierre Alusta1, Dan Buzatu1, Anna Williams1, Willie-Mae Cooper1, Olga Tarasenko2, R. Cameron Dorey1, Reggie Hall3, W. Ryan Parker4 and Jon G. Wilkes1*

1Innovative Safety Technologies Branch, Systems Biology Div., National Center for Toxicological Research, Food Drug Administration, Jefferson, AR, USA
2University of Arkansas at Little Rock, Department of Biology, Little Rock, AR, USA
3Bionetics Corp., National Center for Toxicological Research, Food Drug Administration, Jefferson, AR, USA
4Department of Chemistry, University of Texas, Austin, TX, USA

RATIONALITY: Rapid sub-species characterization of pathogens is required for timely responses in outbreak situations. Pyrolysis mass spectrometry (PyMS) has the potential to be used for this purpose.

METHODS: However, in order to make PyMS practical for traceback applications, certain improvements related to spectrum reproducibility and data acquisition speed were required. The main objectives of this study were to facilitate fast detection (<30 min to analyze 6 samples, including preparation) and sub-species-level bacterial characterization based on pattern recognition of mass spectral fingerprints acquired from whole cells volatilized and ionized at atmospheric pressure. An AccuTOF DART mass spectrometer was re-engineered to permit ionization of low-volatility bacteria by means of Plasma Jet Ionization (PJI), in which an electric discharge, and, by extension, a plasma beam, impinges on sample cells.

RESULTS: Instrumental improvements and spectral acquisition methodology are described. Performance of the re-engineered system was assessed using a small challenge set comprised of assorted bacterial isolates differing in identity by varying amounts. In general, the spectral patterns obtained allowed differentiation of all samples tested, including those of the same genus and species but different serotypes.

CONCLUSIONS: Fluctuations of ±15% in bacterial cell concentrations did not substantially compromise replicate spectra reproducibility. © 2015 National Center for Toxicological Research. Rapid Communications in Mass Spectrometry published by John Wiley & Sons Ltd.

Mass spectrometric analysis of biomolecules can be labor-intensive. Nevertheless, if one regards bacteria grown under standard conditions as a well-defined mixture of chemicals, then mass spectrometry should be applicable for classifying isolates of such bacteria based on the spectral patterns produced.

There is a demand for rapid, reproducible microbiology methods that use easy-to-operate instrumentation, are amenable to automation, and have a high discriminatory power capable of identifying and characterizing microorganisms. Traditionally, numerous wet-lab procedures have been used for taxonomic classification and identification of bacteria. These labor-intensive methods often begin with selective enrichment and isolation to form a pure colony. These methods include growth on target-specific solid media followed by analysis based on distinctive phenotypic traits such as colony morphology and physiological or biochemical test results. Current pathogenic bacteria characterization techniques require a minimum of 48 h, including enrichment and isolation. More modern detection and distinction methods have been developed and include pulsed field gel electrophoresis (PFGE) and genome-wide association studies (GWAS). However, these methods also require colony isolation prior to analysis, are technically demanding, have high unit analysis costs, and afford variable degrees of taxonomic strength. Therefore, there is a demand for rapid, reproducible microbiology methods that use easy-to-operate instrumentation, are amenable to automation, and have a high discriminatory power capable of identifying and characterizing microorganisms.

* Correspondence to: J. G. Wilkes, Innovative Safety Technologies Branch, Div. of Systems Biology, National Center for Toxicological Research (FDA), Bldg. 26, 3900 NCTR Rd., Jefferson, AR 72079, USA.
E-mail: jon.wilkes@fda.hhs.gov

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Rapid Commun. Mass Spectrom. 2015, 29, 1961–1968
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7299
In principle, a system for dependable classification of bacteria should: (1) detect and characterize bacterial pathogen samples reproducibly with high throughput, (2) be able to produce a dynamic relational database, and (3) be easily accessible. Mass spectrometry (MS) offers features that lend it to fulfilling these criteria. Researchers have been trying to achieve that goal for decades, starting with Anhalt and Fenselau who applied electron ionization (EI)-MS for chemotaxonomy purposes as early as 1975.[7]

Prior to analyzing whole cells using mass spectrometry, researchers often analyzed protein extracts and lyzed cells. Heller et al. achieved bacterial profiling by fast atom bombardment (FAB)-MS using lyzed bacterial cells.[8] Ryzhov et al. lyzed bacterial cells and identified biomarkers using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS.[9] Demirev et al. successfully identified bacteria in 1999 based on their protein extracts using MALDI-TOF-MS.[10]

In 1996, Holland et al. identified bacteria using spectral patterns of whole bacterial cells acquired by MALDI-TOF-MS.[11] Basile et al. differentiated microorganisms by their Gram-type using pyrolysis mass spectrometry (PyMS) of extracted fatty acid methyl esters from bacteria.[12] Bacteria were identified on a species level through a certain number of strain-specific metabolites using MALDI-TOF-MS, known as MALDI-based biomarker identification.[13] Miketova et al. successfully Glyphed Gram-type differentiated microorganisms using whole cells.[14] In 2008, Barbuddhe et al. rapidly typed Listeria species based on biomarker identification using MALDI-TOF-MS.[15] Efforts were made to shorten the time required to identify bacteria. Numerous researchers acquired mass spectra of bacteria in 10 min using MALDI-TOF-MS.[16] Mellman et al. succeeded in identifying bacterial species in under 10 min.[17]

Analytical speed was improving but reproducibility in the patterns was another priority. The earliest work with PyMS used EI.[18–22] Potential advantages of this approach included the use of specially compiled mass spectral libraries yielding sufficient reproducibility in relative intensities. Intensity could then become part of a pattern and thereby provide increased specificity.

This method of taxonomic classification of bacteria, or “operational fingerprinting”, would not require identification of unique biomarkers for every strain. Distinction could be obtained based on reliable patterns of relative ion intensity.

The purpose of this study was to show how a variant form of PyMS, namely Plasma Jet Ionization (PJI), could be used to acquire reproducible spectra of whole bacteria cells in under 2 s.[23,29] The speed of acquisition is based on PJI combined with a sample holder of low thermal mass and an atmospheric pressure ion source. The technique is low cost (~$57/sample, labor cost included) and sample preparation is limited to adjusting bacterial cell concentrations.

Unlike MALDI-TOF-MS, PJI allows reliable acquisition of reproducible spectra where the ratios of peak intensities are conserved for a particular bacterial isolate. This reproducibility allows bacteria classification on a strain level without recourse to computationally costly artificial neural networks (ANNs). Goodacre and Kell[24] have reported the successful discrimination of microbial strains using ANNs. Although ANNs have the capability to discover any conceivable interaction between predictor variables, and the capability to indirectly discover multifaceted non-linear relationships between independent and dependent variables, they are predisposed to overfitting, and are computationally costly.

Previously, Meuzelaar and Kistemaker worked with a heating element based on Curie-point pyrolysis in order to improve spectrum reproducibility by reducing variability in the heat transfer process.[25] Others have devised ionization methods based on plasma, a similar concept but with physical configurations and consequent gas-phase ionization processes differing significantly from those presented here.[26–27] Pierce et al. reported using DART for the generation of fatty acid methyl ester (FAME) ions from whole bacterial cells.[28] Although FAME mass spectra were acquired using this simple method (analysis time: 9 min), co-deposition of intact bacterial cells and a tetramethylammonium hydroxide (TMAH) solution was required. Gharaibeh and Voorhees[29] have succeeded in indirectly classifying bacteria by profiling extracted FAMEs. Sample preparation, however, involved an extraction using supercritical CO2. Cheung et al.[30] have reported efficacious discrimination of bacteria using Py-GCDMS, followed by principle component analysis (PCA) and partial least squares (PLS). The use of PLS is accompanied by a risk, specifically when a great number of variables and small sample sizes are involved. The opportunities of confusing classifications and overfitting do exist.

**EXPERIMENTAL**

The mass spectrometer used in this study was an AccuTOF-DART (Direct Analysis in Real Time) manufactured by JEOL USA, Inc. (Peabody, MA, USA). DART sample ionization is based on metastable atom bombardment (MAB)[31] The manufacturer suggests inserting a sample holder containing the analyte into the sample introduction chamber between the DART ion gun and ion entrance orifice for analysis of organic compounds. Although this method suffices for qualitative analysis of volatile organic compounds, it is unsuitable for low-volatility components such as those prominent in bacterial cells.[32] The AccuTOF mass spectrometer was initially re-engineered to perform pyrolysis and ionization of whole bacterial cells using electric sparks delivered through a discharge electrode aiming at the analyte. Consequentially, the AccuTOF mass spectrometer was no longer operating in the DART mode. As far as bacteria are concerned, the ions generated through this discharge are similar to those generated through pyrolysis EI, to MAB, as well as a recently produced new method called DART-Corona independently developed by AMR[33] that also uses a spark generated in a He-enriched atmospheric pressure.

An electric discharge (300μA, 1.98kV, DC) was generated in a continuous manner between the electrically grounded electrode of the ion gun and the high-tension sample holder, so vaporizing and simultaneously ionizing bacterial cells deposited on the sample holder (McMASTER-CARR, Princeton, NJ, USA; Fig. 1 (A)). The required voltage to generate a continuous plasma beam was provided by an external power source (Model CZE1000R, Spellman High Voltage Electronics Corp., Plainview, NY, USA) substituted for the DART’s electronics to prevent compromising the electronic circuitry, which was designed for lower current. Even after using PJI for over three years, damage to these instrumental components has not occurred.
A significant background contamination level persisted in the mass spectra. Some electrons produced in the aforementioned arrangement enter the first stage of the ion optics through the atmospheric pressure sampling port and substantially added to background signal by ionizing oil vapors back streaming from the built-in backing/roughing vacuum pump. Oil residue back-streaming was eliminated by substituting a high-capacity oil-free scroll pump (xD5-10, Edwards) connected to the AccuTOF’s RF-only quadrupole chamber. Since this re-engineered AccuTOF mass spectrometer registers minor differences between spectra, oil vapor chemical noise causes substantial interference.

A sample introduction device (Fig. 1(B)) was designed to implement PJI. It uses lightweight stainless steel wire cloth strips (hereafter referred to as ‘strips’, Fig. 1(A)) as sample holders. Two high-tension copper rods, each terminated with alligator clips A, were attached to a Plexiglas arm, H. The sample-holding arm was hinged to the rest of the device. Strips, holding bacteria in 1-μL indentations (Fig. 1(A)), were secured in position with the alligator clips mounted to the Plexiglas sample-holding arm H of the xyz-stage (Fig. 1(B)). One of the rods was submitted to high-tension (wiring not shown) to establish a continuous and stable plasma beam between the strip and the discharge electrode. The entire sample introduction device was bolted onto the AccuTOF mass spectrometer and adjusted via a xyz-stage to ensure reproducible sample positioning. A slotted borosilicate-glass sleeve, B (Fig. 1(B)), was fabricated and placed between the AccuTOF sample inlet cone and the discharge electrode to ensure a He-rich ionization atmosphere.

Strips (Fig. 1(B)) were fabricated out of commercially available stainless steel wire cloth (100M, x0045 Dia, #12X12CP75; McMASTER-CARR). These electrically conductive strips have a high surface area, similar to that of steel wool, and a small thermal capacity, making them ideal sample holders for locating evaporated samples during spectra acquisition. The strips were indented to hold bacterial suspensions. After fabrication, the strips were sonicated for 15 min each in three different organic solvents (HPLC-grade with low residue after evaporation 1-hexene, acetone, and methanol), subsequently dried and baked at 600 °C for 2–3 h in an electric muffle oven to burn off any remaining organic residues. After cleaning, the strips were only handled by clean metal forceps. They were stored until use in sealed aluminum foil pouches. PJI provides almost universal ionization for organic compounds and is sensitive to minor contamination sources, a significant source of variability when bacterial samples to be distinguished are similar.

Bacterial suspensions are deposited as 1-μL droplets into these indentations (Fig. 1(A)). It was empirically determined that 1 μL is the optimal volume to use based on the size of the indentations, accuracy in dispensing small volume of aqueous suspensions, and the desirably short ambient temperature evaporation time. Another key to spectral reproducibility (Fig. 2) with PJI is the ability to control the number of cells deposited on the sample holder. It was empirically determined that a cell count of ~600,000 cells (±15%) reduces pattern variations attributable to auto-chemical ionization (auto-C.I.) and dramatically increases spectral reproducibility. The ability to rapidly count and deposit 600,000 cells onto a sample holder was facilitated by another technology invented at NCTR, namely RAPID-B™ (Vivione Biosciences, LLC, Dallas, TX, USA). RAPID-B™ is a flow cytometer based system that can count bacteria accurately in real time. This technology was introduced and described in detail in the literature.[34–36] Using the Total Plate Count (TPC) assay on the RAPID-B™ instrument, bacterial suspensions were accurately and rapidly counted. Sample suspensions were then diluted to the required concentration prior to deposition onto strips.

The degree of improvement in reproducibility was quantified by comparing the similarity of replicate spectra from Salmonella enterica subsp. enterica serotype Montevideo when we varied the number of cells deposited on the strip. Spectra of these cells were counted by RAPID-B™, then diluted to concentrations including 600,000 cells/μL and variations about that value. Spectra were acquired by PJI and analyzed for similarity using an NCTR-written spectrum screening software program. The latter computes spectra similarities SS (Eqn. (1)) between two spectra (A and B) based on Euclidian distances. No two replicate mass spectra acquired by PJI from the same microorganism at the same conditions are 100% identical. Slight fluctuations in peak intensities between replicate spectra are to be expected. The adjustment constant C (Eqn.(1)) is determined by the operator during calibration. When replicate spectra of one and the same microorganism are acquired, C is adjusted so that SS will turn out to be above 95%, so characteristic of spectra originating from the same microorganism. The constant C is then fine-adjusted so that SS of spectra acquired from two different microorganisms would fall well below 95%. Typically, the numerical value of constant C gravitates around 4.
The accuracy of the RAPID-B™ counted concentrations was ±2%. Variations of ±15% in bacterial cell concentrations made no obvious difference and spectra were typically similar at a level of 0.97. Quantitative accuracy is needed in analytical microbiology when preparing suspensions in research contexts such as determination of antibiotic resistance or inoculum levels.[37] However, a coefficient of variation smaller than 5% can be obtained for concentrated suspensions using nephelometry or turbidimetry (optical density or clarity measurements).[38] Therefore, flow cytometric quantification is not indispensable to implement this aspect of the PJI process.

We extended the range of variability in the above experiment and found that variations of 2-fold greater and 2-fold lower concentration led to much decreased similarity. We have not seen attempts to control bacterial cell numbers in previous PyMS studies, perhaps because the spectra can always be normalized. In PJI, ionization effects that modify ion ratios defeat normalization. Effective methods therefore must control sample loading, albeit with an error margin of 15%.

After drying, deposited bacteria are not spread out homogenously, but are rather found clumped together in minute crevices found on stainless steel wires of strips (Fig. 3(A)). After being subjected to an electric discharge, the presence of bacteria cannot be confirmed on the strips. Only mineral salts C, crystallized out of the PBS solution, are left behind (Fig. 3(B)). Since mineral salts are more electro-conductive, they appear as brighter particles. This shows that electric discharges consume bacteria, volatilizing their molecular components during PJI. Thus the ion lenses and vacuum system plumbing of the mass spectrometer do not become pathogen contaminated.

One key to spectral reproducibility with PJI is to extract spectra from pyrograms by a consistent rule. Single ion chromatograms at m/z 268 were used to define the range scanned. The first half of the peak was scanned (Fig. 4(B)), thus ensuring objective spectra extraction. The ion at m/z 268 was chosen for spectra extraction due to its relatively higher mass, peak intensity, and ubiquitous occurrence in bacteria. The high mass guaranteed that the ion would not appear by ambient temperature volatilization, but only through PJI.

Spectra reproducibility was demonstrated through the calculation of the Pearson product-moment correlation coefficient, or simply the correlation coefficient, denoted $r$, ranging from 0 for the least similar, to +1 for the most similar spectra. A total of 20 replicate spectra were acquired from Salmonella Bareilly bacterial cells. Spectra were then stored, converted and binned to unit resolution. As many as 14 outliers were identified using OMNIPrint_OW – an outlier wizard program based on distance calculations (i.e., Canberra, Cosine, Manhattan, Pearson, Bray, Curtis, Tanimoto and Jaccard) – and subsequently omitted from the spectra library. Correlation coefficients $r$ were calculated (Eqn. (2)) for each pair of spectra. Results are

$$SS = 100 - (100 \times \sum_{i=1}^{n} (y_{Ai} - y_{Bi})^2) - (C \times \sqrt{\frac{\sum_{i=1}^{m} (|y_{Ai} - y_{Bi}|)^2 - (\sum_{i=1}^{m} |y_{Ai} - y_{Bi}|)^2/n}{n}})$$

where $SS$ is the degree of similarity (%) between spectra $A$ and $B$, $y_{Ai}$ = peak height at $i^{th}$ data point in spectrum $A$, $y_{Bi}$ = peak height at $i^{th}$ data point in spectrum $B$, $n$ = number of ions (unit resolution) contained in a spectrum, and $C$ = adjustment constant (to be determined during calibration).

Most replicate spectra (98%), regardless of whether they were acquired from diluted (510,000 cells/mL) or concentrated (690,000 cells/mL) bacterial cell suspensions, exhibited SS well above the 95% threshold (Fig. 2). Spectra quality degraded substantially below this SS threshold, leading to increasingly inaccurate spectra identifications (data not shown). The 95% SS threshold was determined empirically.

Figure 2. Comparison of two spectra acquired from Salmonella enterica subsp. enterica serotype Montevideo. The top spectrum was acquired from a 15% more dilute cell suspension (510,000 cells/mL), whereas the bottom spectrum was acquired from a 15% more concentrated cell suspension (690,000 cells/mL). Spectra similarity was still above 95%.
summarized in Table 1. The average coefficient of correlation was slightly above 0.95, hinting at reproducibility of spectra acquired through PJI.

\[
r = \frac{n \sum xy - \sum x \sum y}{\sqrt{n \sum x^2 - (\sum x)^2} \sqrt{n \sum y^2 - (\sum y)^2}} (2)
\]

where \(x_i\) and \(y_i\) are intensities along spectra \(x\) and \(y\), respectively. Replicate spectra were acquired from an assortment of ten bacterial isolates (Table 2). The assortment includes different species such as *Vibrio vulnificus* and *Escherichia coli* O157:H7 to demonstrate bacteria characterization on a genus-level, closely related *Bacillus* strains to demonstrate the ability to distinguish species-level (e.g., non-pathogen *B. cereus* from the genetically similar pathogen *B. anthracis*) as well as four different *Salmonella* serovars, all pathogens, to demonstrate bacteria characterization on a serovar-level.

With the exception of the *B. anthracis* Sterne strain, which originates from the Colorado Serum Company (Denver, CO, USA), the other aforementioned bacterial isolates (Table 2)
were all procured from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). All isolates were re-grown overnight on Tryptic Soy Agar (TSA) plates at 37°C, suspended the following day in PBS 1× and purified in a three-wash cycle using centrifugation (8000 rpm). As part of the data processing, all raw profile spectra were compressed into unit resolution.

The compressed spectra were then normalized to the total ion intensity prior to unsupervised hierarchical cluster analysis (HCA, using Origin v.9.0). HCA was used to distinguish spectra acquired of different bacterial species. Results of this HCA are plotted in a dendrogram (Fig. 5).

Spectra of each isolate (Fig. 5) allowed straightforward distinction. Euclidian distances do not directly reflect genetic differences, rather phenotypic differences in spectral silhouettes attributable to expressed genes and based on ions and their fragmentation patterns from biochemicals originating from the bacterial cells. For instance, using HCA, Bacillus anthracis Sterne strain is distinguishable from its genetic cousin Bacillus cereus (genetically 97% identical). Furthermore, distinction was achieved on a serovar-level with Salmonella serovars (i.e., SANE, SAHE, SATY and SAEN). Pattern recognition methods including direct spectral similarity calculations (Euclidian distances), principal component analysis (PCA), hierarchical cluster analysis (HCA), and correlation analysis (data not shown) were applied.[39]

**DISCUSSION**

Replicate spectra were acquired from a small assortment of bacterial isolates out of a larger collection. Spectra of each isolate cluster well in HCA, permitting straightforward bacterial classification. It is interesting to note that the Bacillus anthracis Sterne strain (BAST) is well separated from its genetic cousin Bacillus cereus (BACE). BAST is 97% genetically homologous to BACE.[6] The latter is the microorganism most easily mistaken for BAST.[6] Furthermore, distinction was achieved at a sub-species level as shown with Salmonella serovars (i.e., Salmonella enterica sbsp. enterica serovar Enteritidis (SAEN), Salmonella enterica sbsp. enterica serovar Heidelberg (SAHE), Salmonella enterica sbsp. enterica serovar Newport (SANE), and Salmonella enterica sbsp. enterica serovar Typhimurium (SATY)). With the exception of SANE, each of the other Salmonella serovars appeared in a clade of its own replicates. This is indicative of potential sub-species level distinction. Should a bacterial sample of an unknown origin be tested in the same manner, it would be screened for close matches in a large library of known bacteria spectra. The

| Spectrum | #3 | #5 | #6 | #14 | #16 | #21 |
|----------|----|----|----|-----|-----|-----|
| #3       | 1.0000 | 0.9835 | 0.9823 | 0.9519 | 0.9465 | 0.9574 |
| #5       | 0.9835 | 1.0000 | 0.9875 | 0.9530 | 0.9575 | 0.9290 |
| #6       | 0.9823 | 0.9875 | 1.0000 | 0.9320 | 0.9302 | 0.9331 |
| #14      | 0.9519 | 0.9530 | 0.9320 | 1.0000 | 0.9828 | 0.9433 |
| #16      | 0.9465 | 0.9575 | 0.9302 | 0.9828 | 1.0000 | 0.9291 |
| #21      | 0.9574 | 0.9290 | 0.9331 | 0.9433 | 0.9291 | 1.0000 |
| Average  | 0.9533 ± 0.022 |

**Table 2.** Assortment of bacterial isolates used for bacteria characterization on a genus-, species-, and serovar-level

| #     | Description of microorganism           | Abbreviation |
|-------|----------------------------------------|--------------|
| 1     | Bacillus cereus (ATCC 14579)           | BACE         |
| 2     | Bacillus anthracis Sterne strain (avirulent) | BAST        |
| 3     | Bacillus subtilis (ATCC 6633)          | BASU         |
| 4     | Bacillus thuringiensis (ATCC 35866)    | BATH         |
| 5     | Escherichia coli O157:H7 (ATCC 43888)  | ESCC         |
| 6     | Salmonella enterica sbsp. enterica serovar Enteritidis (ATCC 711671) | SAEN |
| 7     | Salmonella enterica sbsp. enterica serovar Heidelberg (ATCC 8326) | SAHE |
| 8     | Salmonella enterica sbsp. enterica serovar Newport (ATCC 6962) | SANE |
| 9     | Salmonella enterica sbsp. enterica serovar Typhimurium (ATCC 19585) | SATY |
| 10    | Vibrio vulnificus (ATCC 33148)         | VIVU         |

Figure 5. Dendrogram plot (Ward method, using Origin v.9.0) of replicate spectra acquired in triplicate from an assortment of bacterial isolates (Table 2).
dendrogram is an example of unsupervised pattern recognition and the grouping of replicate analyses for species level distinction is evident. The potential for the analytical method described above reflects the inherent taxonomic strength of the PJI-generated spectral patterns.

**CONCLUSIONS**

The modified instrumentation and technique developed in this study allows rapid, facile and reproducible acquisition of mass spectra from whole bacterial cells, and fulfills six of the eight requirements posed by Sauer et al. for dependable identification of bacteria, namely: provide universal bacterial identification capability; detect previously uncharacterized bacteria; find the most closely related bacterial species; enable high-throughput analysis; ensure low operational costs; and rapid transfer of information.[3]

Because the spectrum relative intensities change with bacterial cell concentrations, quantifying an unknown bacterium (Sauer requirement #2, i.e., quantitative analysis) by PJI cannot be done directly without counting bacterial cells, presumably due to auto-C.I. Highly concentrated samples lead to massive fragmentation and whole envelopes of peaks that obscure the biomarkers used to identify microbial samples. Assigning any chemical significance to any peaks of biomarkers observed in mass spectra acquired of bacterial cells remains a daunting task. The RAPID-B™ system is designed for sensitive detection and quantification of bacterial cells. The last requirement of Sauer et al., i.e., sharing spectral data through readily accessible and regularly updated databases, is possible due to PJI pattern consistency.[3] A demonstration of library building and consulting is part of future work and should be imminent now that the overall system design, instrumental hardware modifications, and data processing issues have been satisfactorily resolved.

The technique developed in this study has the following characteristics:

- Rapid (sample preparation: 2–5 min., duplicate spectra acquisition: 10 s, data preprocessing: 15 s, spectra identification: 1–3 min);
- Affordable (a maximum of $2 each for the strips, ~$15 in reagents for counting cell suspension concentrations by RAPID-B™, and $40 in labor cost); and
- Reliable (bacterial identification at sub-species level).

Prospective work consists of refining spectral data handling to enable bacterial identification at a serovar-, sub-serovar-, or near-isolate-level and building of a large, standardized spectrum library.

**Acknowledgements**

This study conforms to the IBC protocol # 09092. The authors extend their gratitude to ORISE for partial financial support. The opinions expressed are those of the authors and do not necessarily reflect those of the USFDA.

**REFERENCES**

[1] B. M. Ham. Even electron mass spectroscopy with biomolecule applications. John Wiley, New York, 2008, pp. 41–42.

[2] S. Vaidyanathan, D. B. Kel, R. Goodacre. Flow-injection electrospray ionization mass spectrometry of crude cell extracts for high-throughput bacterial identification. J. Am. Soc. Mass Spectrom. 2002, 13, 118.

[3] S. Sauer, M. Kliem. Mass spectrometry tools for the classification and identification of bacteria. Nat. Rev. Microbiol. 2010, 8, 74.

[4] T. Ezaki. Genetic identification of bacterial pathogens. Nihon Rinsho 1994, 52, 355.

[5] T. A. Pearson, T. A. Manolio. How to interpret a genome-wide association study. J. Am. Med. Assn. 2008, 299, 1335.

[6] W. Joklik, H. Willett, B. Amos, C. Willert. Zinsser Microbiology. (20th edn.). Appleton & Lange, Norwalk, CT, 1992, pp. 615–619.

[7] J. P. Anhalt, C. Fenselau. Identification of bacteria using mass spectrometry. Anal. Chem. 1975, 47, 219.

[8] D. N. Heller, R. J. Cotter, C. Fenselau. Profiling of bacteria by fast atom bombardment mass spectrometry. Anal. Chem. 1987, 59, 2806.

[9] V. Ryzhov, Y. Hathout, C. Fenselau. Rapid characterization of spores of Bacillus cereus group bacteria by matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry. Appl. Environ. Microbiol. 2000, 66, 3828.

[10] P. A. Demiere, Y. P. Ho, V. Ryzhov, C. Fenselau. Microorganism identification by mass spectrometry and protein database searches. Anal. Chem. 1999, 71, 2732.

[11] R. D. Holland, J. G. Wilkes, F. Rafii, J. B. Sutherland, C. C. Persons, K. J. Voorhees, J. O. Lay Jr. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 1996, 10, 1227.

[12] F. Basile, K. J. Voorhees, T. L. Hadfield. Microorganism gram-type differentiation based on pyrolysis-mass spectrometry of bacterial fatty acid methyl ester extracts. Appl. Environ. Microbiol. 1995, 61, 1534.

[13] R. D. Holland, C. R. Duffy, F. Rafii, J. B. Sutherland, T. M. Heinze, C. L. Holder, K. J. Voorhees, J. O. Lay Jr. Identification of bacterial proteins observed in MALDI TOF mass spectra from whole cells. Anal. Chem. 1999, 71, 3226.

[14] P. Miketova, C. Abbas-Hawks, K. J. Voorhees, T. L. Hadfield. Microorganism gram-type differentiation of whole cells based on pyrolysis high-resolution mass spectrometry data. J. Anal. Appl. Pyrolysis 2003, 67, 109.

[15] S. B. Barbuuddhe, T. Maier, G. Schwarz, M. Kostrzewa, H. Hof, E. Domann, T. Chakraborty, T. Hain. Rapid identification and typing of listeria species by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Appl. Environ. Microbiol. 2008, 74, 5402.

[16] T. Krishnamurthy, P. L. Ross. Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells. Rapid Commun. Mass Spectrom. 1996, 10, 1992.

[17] A. Mellmann, J. Cloud, T. Maier, U. Keckevoet, I. Ramming, P. Iwen, J. Dunn, G. Hall, D. Wilson, P. LaSala, M. Kostrzewa, D. Harmsen. Evaluation of matrix-assisted laser desorption/ionization-time of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. J. Clin. Microbiol. 2008, 46, 1946.

[18] P. R. Sisson, R. Freeman, F. K. Gould, N. F. Lightfoot. Strain differentiation of nosocomial isolates of Pseudomonas aeruginosa by pyrolysis mass spectrometry. J. Hosp. Infect. 1991, 19, 137.

[19] P. R. Sisson, R. Freeman, N. F. Lightfoot, I. R. Richardson. Incrimination of an environmental source of a case of Legionnaires’ disease by pyrolysis mass spectrometry. Epidemiol. Infect. 1991, 107, 127.

[20] P. R. Sisson, R. Freeman, J. G. Magee, N. F. Lightfoot. Differentiation between mycobacteria of the Mycobacterium
tuberculosis complex by pyrolysis mass spectrometry. *Tubercle* 1991, 72, 206.

[21] P. R. Sisson, R. Freeman, J. G. Magee, N. F. Lightfoot. Rapid differentiation of *Mycobacterium xenopi* from mycobacteria of the *Mycobacterium avium-intracellular* complex by pyrolysis mass spectrometry. *J. Clin. Pathol.* 1992, 45, 355.

[22] P. R. Sisson, J. M. Kramer, M. M. Brett, R. Freeman, R. J. Gilbert, N. F. Lightfoot. Application of pyrolysis mass spectrometry to the investigation of outbreaks of food poisoning and non-gastrointestinal infection associated with Bacillus species and Clostridium perfringens. *Int. J. Food Microbiol.* 1992, 17, 57.

[23] P. Alusta, J. Wilkes, D. Buzatu, C. Dorey, C. Dorey, W. M. Cooper. Discrimination of bacteria using pyrolysis-gas chromatography-differential mobility spectrometry (Py-GC-DMS) and chemometrics. *Analyst* 2009, 134, 557.

[24] R. B. Cody, J. A. Laramée, H. D. Durst. Versatile new ion source for the analysis of materials in open air under ambient conditions. *Anal. Chem.* 2005, 77, 2297.

[25] Available: www.amr-inc.co.jp/product/analysis/DART-corona++.pdf.

[26] J. G. Wilkes, R. K. Tucker, J. A. Montgomery, W. M. Cooper, J. B. Sutherland, D. A. Buzatu. Reduction of food matrix interference by a combination of sample preparation and multi-dimensional gating techniques to facilitate rapid, high sensitivity analysis for *Escherichia coli* serotype O157 by flow cytometry. *J. Food Microbiol.* 2012, 30, 281.

[27] D. A. Buzatu, W. M. Cooper, C. Summage-West, J. B. Sutherland, F. Rafii, A. J. Williams, D. A. Bass, L. D. Smith, R. S. Woodruff, J. M. Christman, S. Reid, R. T. Tucker, C. J. Haney, A. Ahmed, J. G. Wilkes. Photobleaching with phloxine B sensitizer to reduce food matrix interference for detection of *Escherichia coli* serotype O157:H7 in fresh spinach by flow cytometry. *Food Microbiol.* 2013, 36, 416.

[28] D. A. Buzatu, T. J. Moskal, A. J. Williams, W. M. Cooper, W. B. Mattes, J. G. Wilkes. An integrated flow cytometry-based system for real-time, high sensitivity bacterial detection and identification. *PLoS One* 2014, 9, e94254.

[29] S. Sutton. Determination of inoculum for microbiological testing. *Journal GXP Compliance* 2011, 15, 49.

[30] L. L. Smith, R. S. Woodruff, J. M. Christman, S. Reid, R. T. Tucker, C. J. Haney, A. Ahmed, J. G. Wilkes. Photobleaching with phloxine B sensitizer to reduce food matrix interference for detection of *Escherichia coli* serotype O157:H7 in fresh spinach by flow cytometry. *Food Microbiol.* 2013, 36, 416.

[31] G. C. Conway, S. C. Smole, D. A. Sarracino, R. D. Arbet, P. E. Leopold. Phyloproteomics: species identification of enterobacteriaceae using matrix assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mol. Microbiol. Biotechnol.* 2001, 3, 103.