Detection of *Pseudomonas aeruginosa* biomarkers from thermally injured mice in situ using imaging mass spectrometry

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Monitoring patients with burn wounds for infection is standard practice because failure to rapidly and specifically identify a pathogen can result in poor clinical outcomes, including death. Therefore, a method that facilitates detection and identification of pathogens in situ within minutes of biopsy would be a significant benefit to clinicians. Mass spectrometry is rapidly becoming a standard tool in clinical settings, capable of identifying specific pathogens from complex samples. Imaging mass spectrometry (IMS) expands the information content by enabling spatial resolution of biomarkers in tissue samples as in histology, without the need for specific stains/antibodies. Herein, a murine model of thermal injury was used to study infection of burn tissue by Pseudomonas aeruginosa. This is the first use of IMS to detect P. aeruginosa infection in situ from thermally injured tissue. Multiple molecular features could be spatially resolved to infected or uninfected tissue. This demonstrates the potential use of IMS in a clinical setting to aid doctors in identifying both presence and species of pathogens in tissue.

Burn injuries are one of the most common forms of trauma and represent a major health concern as burn-related infections are a sig-nificant cause of morbidity and mortality [1,2]. In the United States alone, there are approximately 1.2 million burn injuries each year, primarily among young children and the elderly [3]. Roughly 100,000 of these injuries are classified as severe, requiring hospitalization, and five percent result in death of the patient [3,4]. The average cost to treat a hospitalized burn patient is $200,000, and it is estimated that$18 billion is spent treating burn patients in the United States alone each year [3,5]. Compounding the economic burden of burn injuries is the increased risk of wound infection and sepsis, the primary cause of mortality in burn patients [6–8].

Under normal circumstances, healthy skin tissue acts as a natural barrier to microbes. However, in burn injuries this function is compromised, providing pathogens an opportunity to establish an in-fection in the patient. Pseudomonas aeruginosa is one of the most common bacterial isolates recovered from burn wounds and is asso-ciated with the highest mortality rate in burn patients [9,10]. This is attributed to the fact that P. aeruginosa is equipped with a battery of virulence factors that facilitate rapid colonization and dissemination when the epithelial barrier is compromised, as is observed in patients with severe burns [11–13]. The progression from skin to vascular in-fection and eventual sepsis has been shown to be as rapid as 24 h [14]. Thus, P. aeruginosa poses a serious threat to patients with impaired defense mechanisms, and early detection techniques are critical to re-duce the mortality rates stemming from sepsis.

Antimicrobial agents can be used to treat patients with P. aeruginosa infections, but the infections can be difficult to detect in a timely fashion with current methodologies. Despite the prevalence of pathogens such as P. aeruginosa in burn injuries, currently there are no rapid

techniques for *in situ* detection, identification, or assessment of distribution in a clinical setting [15]. Technical limitations in the evaluation of potentially infected tissue from clinical samples currently prevent broad and robust *in situ* detection and identification of pathogens. Current methods for the detection of pathogens require sampling of a patient's wound by either swab or tissue biopsy [3]. Identification of burn wound pathogens is routinely performed using microbial culture techniques in the clinical microbiology laboratory, but novel methods including nucleic acid detection and antigen detection have been explored [3,16,17]. A major problem with these techniques is that the time required from biopsy to identification is often 24 h or more [3]. Identifying and differentiating infecting organisms while preserving their *in situ* geography using histopathological techniques, such as fluorescence *in situ* hybridization or immunohistochemistry, is not a routine procedure for burn tissue as these techniques are limited because they detect only one or a few proteins or nucleic acid sequences at a time [3,17].

The use of matrix assisted laser desorption/ionization (MALDI) mass spectrometry (MS) is now routine for the identification of cultured bacteria in clinical microbiology [18,19]. However, cultivation of isolated organisms is required before MALDI MS can be used for routine pathogen identification. Along similar lines, MALDI imaging MS (MALDI-IMS) has been used to investigate biomarkers *in situ*. To date, this has been practiced strictly outside of the clinic. By rastering a laser across the tissue, mass spectra can be collected in an array. The ion intensities for individual mass-to-charge ratios are then visualized in a two dimensional image as a heat map [20]. However, there have not been any attempts in the literature to detect microorganisms or tissue infection directly from infected tissue sections using MALDI-IMS. Recently, we demonstrated the use of MALDI-IMS to investigate bacteria and antimicrobial agent distribution in an *in vitro* model [21]. In the current study, we applied MALDI-IMS to the *in situ* detection of *P. aeruginosa* infecting a burn wound using a well-studied thermal-injury mouse model [13,22,23]. This is the first report of detecting a pathogen *in situ* using MALDI-IMS directly from tissue sections.

Methods

Bacterial strains and growth conditions

P. aeruginosa strain PAO1 was used in this study [24]. Cultures of *P. aeruginosa* were grown aerobically in lysogeny broth (LB) at 37 °C, with shaking at 200 revolutions per minute (rpm). An aliquot of this overnight growth was transferred to fresh LB and incubated for 3 h at 37 °C with shaking at 200 rpm.

Mouse model of thermal injury

The thermally-injured mouse model was adapted from the burned-mouse model protocol described by Stieritz and Holder [14,25,26]. Burn experiments were conducted in adult female Swiss Webster mice (Charles River Laboratories) weighing between 20 and 25 g. The mice were anaesthetized by intraperitoneal injection of 0.4 ml Nembutal at 5 mg/ml (5% sodium pentobarbital; Oak Pharmaceuticals, Inc.) before their dorsum was shaved, and the hair cleanly removed with a depilatory agent (Nair™). The mice were then securely placed into a template with an opening (4.5 cm × 1.8 cm) designed to expose approximately 15% total body surface area (TBSA) of the animal's dorsum to a thermal water bath. Thermal injury was induced by placing the exposed area of the shaved skin in 90 °C water for 10 s. This scald injury is non-lethal but induces a third-degree (full-thickness) burn. Immediately following thermal insult, mice received fluid replacement therapy via subcutaneous injection of 800 μL Lactated Ringer's solution into the nape of the neck. Mice were then inoculated with a subcutaneous challenge of approximately 10^2 cells of *P. aeruginosa* in 100 μL phosphate-buffered saline (PBS) or 100 μL of PBS without bacteria. Where specified, non-injured mice served as a control and their backs were shaved and treated with a depilatory agent in parallel with thermally-injured mice. At 40 h post burn and 5 days post burn, mice were euthanized by intracardiac injection of 200 μL (390 mg/mL) Fatal-Plus® (Vortech Pharmaceuticals, LTD.). Animals were treated humanely and in accordance with protocol #96020 approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center in Lubbock, Texas.

Preparation of P. aeruginosa inoculum for challenge in thermally-injured mice

P. aeruginosa inoculum for challenge in thermally injured mice were prepared as previously described [14,26]. Briefly, an aliquot from an overnight culture of *P. aeruginosa* was subcultured in fresh LB for approximately 3 h at 37 °C, and then adjusted to an OD_{590} of 0.9 using sterile 1X PBS. After O.D. adjustment, a 1 mL aliquot of the subculture was pelleted, washed (twice) in 1X PBS, and then serially diluted (10-fold dilutions) in sterile 1X PBS. A 100 μL aliquot of the 10^{-5} dilution was used to inoculate each animal via subcutaneous injection directly into the burn wound. This dilution has previously been determined to contain approximately 2×10^2 to 3×10^2 colony forming units (CFU) of *P. aeruginosa*; this dose of the wild-type *P. aeruginosa* strain, PAO1, has been shown to cause 94 to 100% mortality in Swiss Webster mice by 48 h post burn and infection [26]. The number of CFU in the pre-injection inoculum was verified by plating 100 μL of the inoculum on *Pseudomonas* isolation agar (BD Difco™).

Collection and sectioning of tissue samples

The embedding medium, 2% carboxymethyl cellulose (CMC) plus 5% gelatin, was selected because it is compatible with MALDI-IMS. This was prepared immediately prior to tissue collection. Briefly, CMC sodium salt (medium viscosity) (MP Biomedicals, LLC.) and 5% gelatin type A (ACROS Organics™) were combined in water in a 50 mL conical tube and heated in a standard microwave with intermittent stirring. The CMC + gelatin embedding mixture was then held at 65 °C in a water bath. Individual tissue samples resected from the dorsum of burned mice (with or without infection) were resized to approximately 1 cm × 0.5 cm and positioned in a Tissue-tek® vinyl specimen Cryomold® (Sakura Finetek) containing CMC + gelatin embedding medium and then placed into a −80 °C freezer to solidify before sectioning (see Fig. 1 for a schematic representation of re-orientation and sectioning procedures). Tissue from non-burned mice was resected from the center of the dorsum and prepared in parallel with burned tissue samples. Frozen samples embedded in CMC + gelatin were sectioned using an OTF5000 cryostat (Bright Instrument Co Ltd.) to a thickness of 20 μm and then directly transferred to a glass slide with an indium-tin oxide (ITO) coated surface (HTX Technologies). A positive control of *P. aeruginosa* culture was spotted onto the slide. Prepared slides were stored at −80 °C until matrix application.

Matrix application for MALDI-IMS

Matrix application has been previously described [21]. Briefly, tissue sections on ITO glass slides were dehydrated in a desiccator for 30 min. A solution of 150 mg/mL 2,5-dihydroxybenzoic acid (DHB, Sigma) in 50% aqueous methanol and 0.05% formic acid (Sigma) was applied using an airbrush held approximately 25 cm from the slide. A spray time of 30 s with one minute of drying time between was used until 15 mL of matrix solution had been dispensed (roughly 15 passes). Slides were then dehydrated in a desiccator for 30 min and then analyzed by MALDI-IMS.

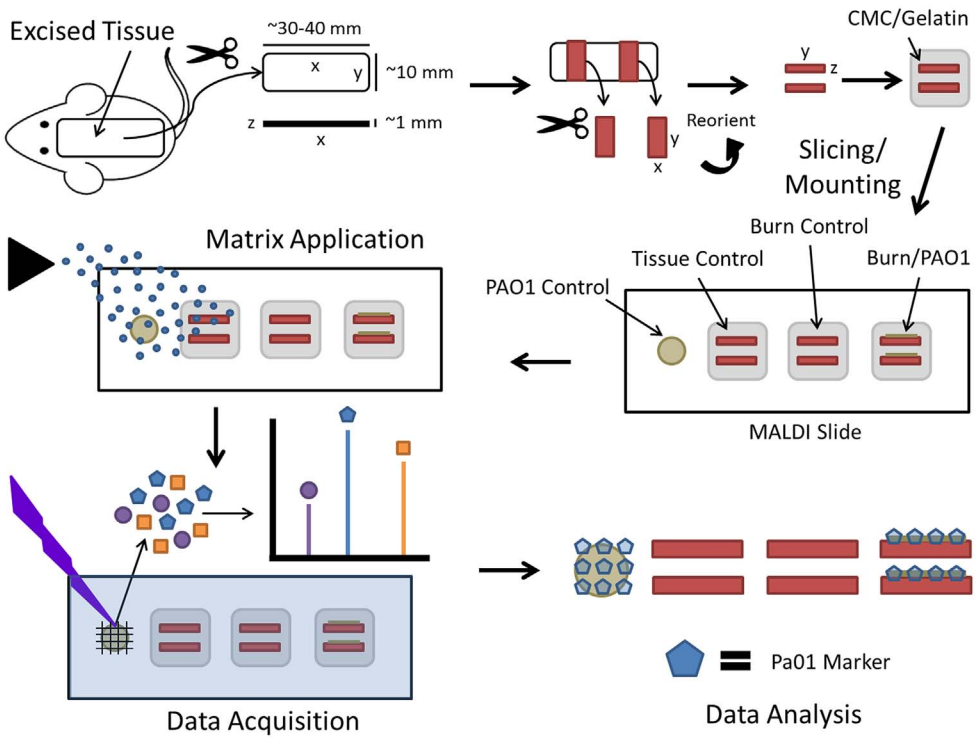

Fig. 1. MALDI-IMS experimental workflow. Tissue from mice (healthy-control, burn-control, and burn-infected) were excised, embedded in carboxymethyl cellulose (CMC)/gelatin media, frozen, and sectioned. Tissue sections along with a control spot of culture PAO1 were placed on indium-tin oxide (ITO) coated glass slides. Slides were coated in 2,5-dihydroxybenzoic acid (DHB) matrix and MALDI-IMS data was acquired. Analysis of data was performed using flexImaging software (Bruker Daltonics) and MSiReader (North Carolina State University).

MALDI-IMS of burn-infected tissue sections

Imaging data was collected using a Bruker Autoflex III MALDI Time-of-Flight (TOF) mass spectrometer (Bruker Daltonics) equipped with a Smartbeam Nd:YAG laser (355 nm). The instrument was operated in positive ionization mode with the TOF in reflection mode. Calibration was performed before each image acquisition using a custom tune mix spotted onto the slide prior to matrix application (see Fig. 2 for slide layout). The custom tune mix consisted of glutamine, histidine, phenylalanine, HEPES, SDS, and CHAPS. For data acquisition, spectra were collected in the mass range 50–1000 m/z by averaging 500 laser shots per pixel with a 150 μm lateral resolution using flexImaging v2.1 (Bruker Daltonics). Representative spectra for two pixels can be seen in Supplemental Fig. 1. Data was exported as an IMG file (.img) from flexImaging and imported into MSiReader v0.06 (North Carolina State University) for visualization of images. The data set was normalized to total ion count. Initially, features which were unique to *P. aeruginosa* were found using the MSiReader peak finding function. Features which were present in the *P. aeruginosa* culture spot but not in the reference area were plotted as heat maps to verify their distribution was unique to *P. aeruginosa*. Additional features were identified by manually generating heat maps, each with the most abundant feature in a 0.5 Da bin window.

Results and discussion

The potential of MALDI-IMS as a diagnostic tool for monitoring infection in a burn wound was assessed using an *in situ* tissue model. A diagram depicting the workflow for MALDI-IMS analysis of infected tissue samples is shown in Fig. 1. MALDI-IMS analysis of burn tissue inoculated with *P. aeruginosa* revealed a number of features that could be used for the *in situ* detection of infection (Table 1). Tentative

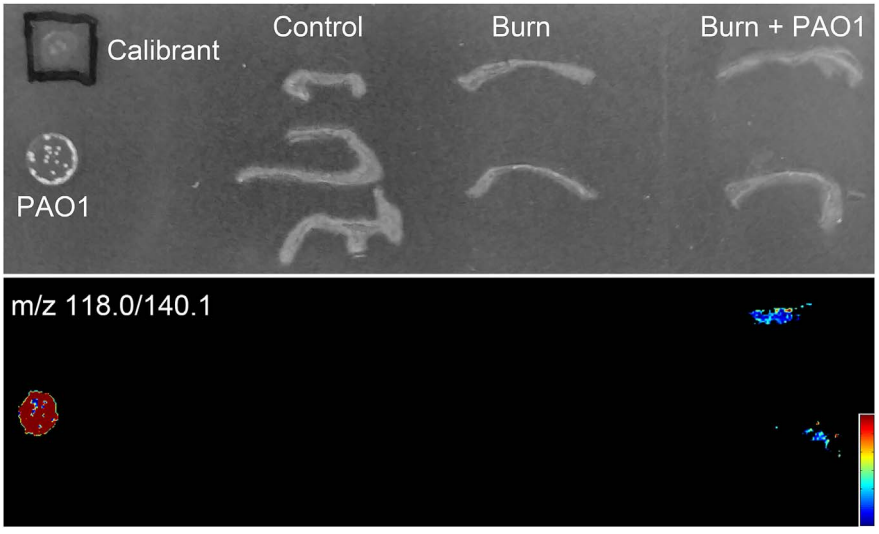

Fig. 2. Optical image and MALDI-IMS heat map for ion 118.0/140.1 m/z from tissue 40 h post infection. Optical image of a slide with sectioned tissue and relevant controls after matrix application (upper panel). A heat map shows the location of ion 118.0/140.1 m/z based on intensity (lower panel). Labels denote samples in upper and lower panels. The scale bar in the lower panel shows the relative intensity of ions from all spectra collected.

Table 1
Molecular features unique to burn-infected tissue.

m/z	Tentative ID	Adduct
74.0	2-iminoacetate	H
	propioamide	H
118.0	L-aspartate-semialdehyde	H
	L-valine[a]	H
	5-aminovalerate	H
140.1	L-aspartate-semialdehyde	Na
	L-valine	Na
	5-aminovalerate	Na
148.1	L-glutamate[a]	H
	L-4-hydroxyglutamate semialdehyde[a]	H
	O-acetyl-L-serine[a]	H
	taurine	Na
	(2-aminoethyl)-phosphenate[a]	H
	5-methylcytosine	H
257.2	β-D-ribosylnicotinate[a]	H
	palmitate	H
272.2	pyridoxine-5′-phosphate	Na
338.1	S-hydroxymethlglutatione	H
367.9	15Z-tetracosenoate	H
	guanosine 2′,3′-cyclic monophosphate	Na
	thiamin phosphate	Na

[a] Denotes metabolites in LC-MS analysis of PAO1.

identifications have been assigned to each unique feature based on mass matches to the Biocyc curated database for *P. aeruginosa* within a range of ± 0.25 Da, which is the limit of accuracy of the MALDI system when imaging tissue sections. Additionally, several features in Table 1 were identified in an untargeted metabolomics analysis of *P. aeruginosa* in our lab using a high mass accuracy quadruple time-of-flight (Q-TOF) mass spectrometer (unpublished data).

The features listed in Table 1 were selected because they are unique to spectra collected from the *P. aeruginosa* control spot and tissue that was infected, suggesting these were metabolic signatures unique to *P. aeruginosa or infected tissue*. A heat map of ion 118.0 m/z illustrates the lack of signal in control tissues and a strong signal in both the *P. aeruginosa* control spot and infected tissue (Fig. 2). Ion 140.1 *m/z* displays a similar distribution in the heat map to that of 118.0 *m/z*. This suggests that these mass-charge ratios represent the same molecular species with a hydrogen ion (118.0 *m/z*) or a sodium ion (140.1 *m/z*) associated with it. The control spot of *P. aeruginosa* shows a higher signal overall compared with tissue sections, because ion signals are coming only from the bacteria, rather than a mixture of *P. aeruginosa* and wound tissue. Heat maps were also generated for the remaining ions in Table 1 (Supplemental Fig. 2).

The molecular features from Table 1 were highly reproducible, consistently appearing in tissue across experimental replicates (Fig. 3). On the left are optical images of DHB coated tissue, where the arrows indicate the orientation of the tissue (see Fig. 1), while the right panels show the heat map generated from the ion 118.0 m/z. The top panels are tissue excised from mice 40 h post inoculation (Fig. 3A and B), while the bottom panels are tissue excised from mice 5 days post inoculation (Fig. 3C and D). Using this mouse model, it has been demonstrated that the bacterial load increases over time, exceeding

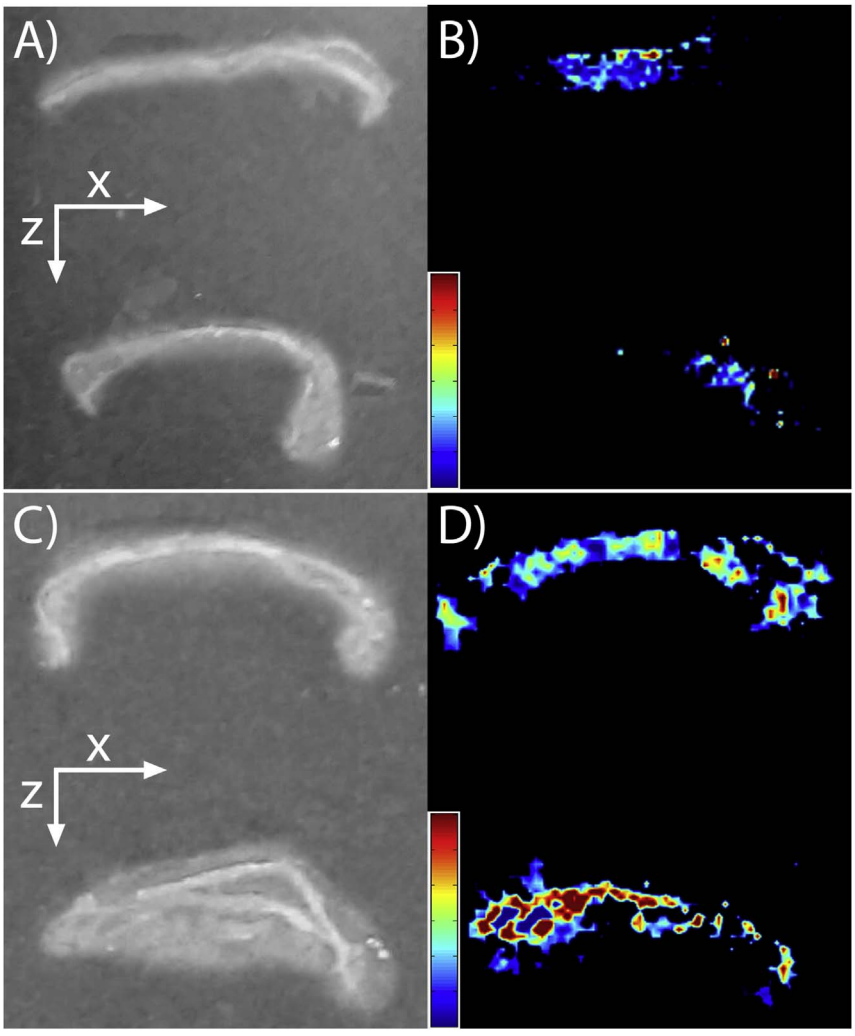

Fig. 3. Analysis of infected tissue. Optical images (A and C) and heat map images showing the ion 118.0 (B and D) acquired from two separate mice during two separate experiments. The arrows show the orientation of the tissue based on the diagram in Fig. 1. The tissue in the upper panels was excised 40 h post inoculation while the lower panels represent tissue that was excised 5 days post inoculation. The heat maps shown in B and D show relative ion intensity, where blue is low and red is high. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

10^9 CFU/g tissue as early as 24 h post burn and infection [13,22,23]. We observed that the relative intensity of the *P. aeruginosa*-specific molecular species also increased over time when examining the relative intensity of these molecules (Fig. 3). This result indicates that MALDI-IMS could potentially be used to quantify the bacteria load present *in situ* [13,22,23].

The ability to spatially resolve ions across a tissue section using MALDI-IMS is this method's hallmark and can be immensely helpful when monitoring the infection of tissue with respect to traditional methods that homogenize samples prior to analysis. A limitation of any analytical method is that low abundance molecular species can be obscured by background signals, or that sporadic noise could result in false positive signals. However, in our data, the signal to noise ratio was excellent for regions which displayed even low signal (blue regions in the heat maps displayed in Fig. 3) relative to the rest of the image. No signal for ions of interest was found in control tissue spectra, while in the infected burn tissue ion intensity was at least five-fold greater than background. While the absolute abundance of the molecular species in the tissue sections was not determined, we have demonstrated that we can detect and spatially resolve sites of bacterial infection in burn tissue.

Conclusions

This is the first report to demonstrate that molecular-level imaging, specifically MALDI-IMS, can be used to delineate tissue infected with bacteria. We identified multiple molecular signals *in situ* that are present exclusively in infected tissue and positive *P. aeruginosa* controls, but are absent in uninfected tissue. The method described here of excising tissue for MALDI-IMS mimics a biopsy of tissue from a patient and does not require lengthy culturing of the pathogen. Additional work needs to be performed in order to determine the composition of the molecular signatures that have been identified, the maximum *m/z* range that could be used when acquiring MALDI-IMS spectra, whether or not polymicrobial infections could be identified to the organism level using MALDI-IMS, and if similar results can be obtained from human samples. We believe our method for the identification of bacterial infection *in situ* demonstrates a new method for detecting bacterial infections directly from infected tissue. This method of identification has the potential to bridge the gap between microbiological culture and histopathological analysis for the infected tissue, thereby aiding in the effective and timely treatment of bacterial infections.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.10.012.

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