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Real time human micro-organisms biotyping based on Water-Assisted Laser Desorption/Ionization

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Abstract

We previously demonstrated that remote infrared Matrix Assisted Laser Desorption Ionization technology (Spidermass) using endogenous water as matrix (or so called water assisted laser desorption/ionization) was enabling real-time *in vitro* and *in vivo* analysis of clinical pathological tissues. In the present work, Spidermass was used to biotype human pathogens either from liquid bacteria growth in time course, from petri dish or on smears. Reproducibility experiments as well as bacteria dispersion and lipids identifications with SpiderMass in MS/MS mode were undertaken. The whole of the data establish that SpiderMass instrument allows real time bacteria biotyping and can be useful in clinic for pathogen identification.

Keywords: Spidermass, real time analysis; mass spectrometry, bacteria typing

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Introduction

Until recently, bacterial identification in clinical laboratories has been mainly relying on conventional phenotypic and gene sequencing identification techniques. The identification of anaerobic bacteria is still fastidious due to slow bacteria growing. Many anaerobes are difficult in most diagnostic systems. For example, 12 weeks are needed for a clear diagnosis of active tuberculosis. This very long-time frame are really challenging for case identification. However, the last decade has seen the introduction of mass spectrometry into clinics for bacteria biotyping (1-4). Bacterial typing by MS is based on the fingerprinting of bacteria ribosomal proteins profiles using Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry (MS) through interrogation of a database of reference profiles (5, 6). The technology was shown to be highly efficient after several inter-laboratory cross-validation(7) and MALDI MS biotyping has become a conventional method for hospital routine biotyping due to its speed, simple handling, cost-effectiveness and high-throughput capabilities(8). The U.S. Food and Drug Administration (FDA) has approved the MALDI Biotyper (Bruker) and the VITEK MS (Biomerieux) (9) bacterial biotyping systems for diagnostic purposes at the Hospital. However, yet some limitations exist in the method. Since the method is based on profiling the most abundant proteins which are constantly expressed (10), the identification rates using clinical isolates are reported to be 79.9% to 93.6% at the species level and 94.5% to 97.2% at the genus level (11, 12). Moreover, some treatment are needed in order to reduce some interferences from blood culture, and bacteria extracts are therefore treated with solvent such as 70% formic acid to extract some molecules out for MALDI-TOF analysis (13). Lower confidence scores sometimes were observed due to occasional polymicrobial samples or lower bacteria number collected from the blood culture, yet MS based method is certainly faster than usual cultured-based approach (13). Thus, MALDI-TOF MS biotyping

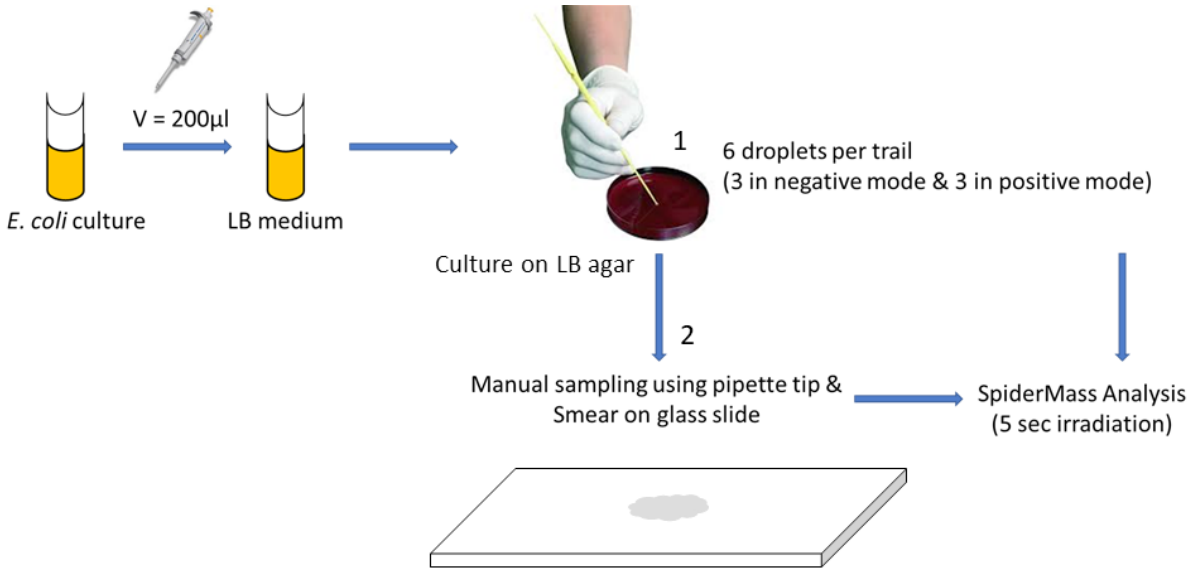


Figure 1. Schematic representation of the tests proceed for pathogen identification using SpiderMass technology. 1) *E. Coli* were cultivated in poor broth medium. Microbial growth was assessed by the increase in A 620 nm after 8-hour incubation at 37°C . At different time of bacteria growth, 10 µl of the suspension are harvested and dropped either on agarose of a petri dish or smeared on glass slides. Direct analyses with SpiderMass were then conducted directly on the top of petri dish or smear. Direct m/z spectra profiles are then recorded.

systems have paved the way to a change of paradigm in bacteria typing. However, higher accuracy, confidence and convenience is still searched for. In this context, ambient ionization mass spectrometry (AIMS) techniques could represent an interesting alternative because of the limited sample preparation requested using such ambient methods are recently introduced in this field (14). Rapid Evaporative Ionisation Mass Spectrometry (REIMS) has been shown to provide a high-throughput platform for the rapid and accurate identification of bacterial and fungal isolates (15). In comparison to MALDI MS commercial systems, REIMS require no preparative steps nor time-consuming cell extractions (15). Species classification accuracy was found ranged between 96%-100% (16).

In this context, we performed human microorganism's bio-typing with a novel ambient ionization mass spectrometer; the Spidermass instrument. The technology is based on Remote Infrared Matrix-Assisted Laser Desorption/Ionization (Remote IR-MALDI) system using tissue endogenous water as matrix (17-19)and was shown to enable in-vivo real-time mass spectrometry analysis with minimal invasiveness in intra-operative conditions during surgery or on ex vivo tissue resection (20).

Material and Methods

Human microorganisms

Gram negative (*E. coli D31, Pseudomonas aeruginosa);* Gram positive (St*aphylococcus aureus, Enterococcus faecalis)* and Yeast (*Candida Albicans*) were cultivated as described previously (21). Briefly, bacteria were cultivated in poor broth medium. Microbial growth was assessed by the increase in A 620 nm after 8-hour incubation at 37°C.

Instrumentation

The basic design of the instrument setup is already described in a previous study (17, 22). In these experiments, the prototype was equipped with a fibered tunable InfraRed Optical parametric oscillator (OPO) system between 2.8 µm to 3.1 µm (Radiant version 1.0.1, OPOTEK Inc., Carlsbad, USA) pumped by a 1.064 µm radiation delivered by a Q-switched 10 ns pulse width Nd:YAG laser (Quantel Laser, Les Ulis, France). A 1 meter length biocompatible laser fiber with 450 µm inner diameter (HP fiber, Infrared Fiber Systems, Silver Spring, USA) was connected to the exit of the OPO system and focused by a 20 mm focal length CaF2 lens attached at its end. A Tygon® ND 100-65 tubing (2.4 mm inner diameter, 4 mm inner diameter, Akron, USA) was used to aspirate the ablated material and was directly connected to the inlet of the mass spectrometer (Synapt G2s, Waters, Manchester) through a modified atmospheric pressure interface described elsewhere (18) (**Fig. 1**). The samples was irradiated over 5 sec at 1.6 J/cm^2. The fluence was decreased to 0.8 J/cm2 when the wavelength was tuned to allow for keeping it constant over the screened range. The spectra acquisition was performed in positive resolution mode with a scan time of 0.5 sec. For the experiments related to the evaluation of the transfer tube temperature, a flexible heating cable system (isopad T7000; Thermocoax, Suresnes, France) was uniformly winded around the aspiration tube.

Special care was taken when working with infrared laser beams. The laser used for this study is classified as safety class IV which requires the wear of specific laser safety goggles throughout all experiments. The samples were irradiated over 5 sec at 1.6 J/cm^2. Only when the wavelength was tuned the

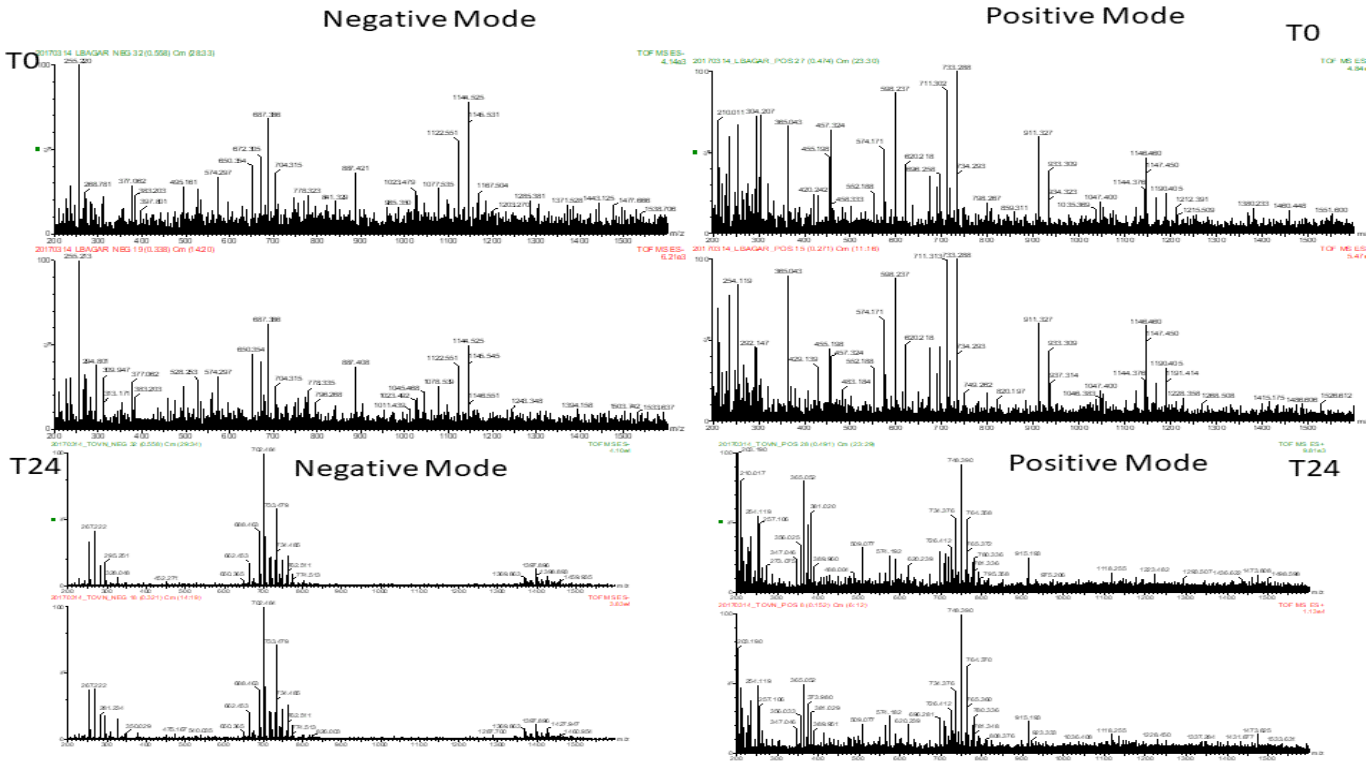


Figure 2. Time course SpiderMass analyses from T0 to T24 hours of *E. Coli* growths in liquid suspension were performed on agarose of a petri dish. I 10 µl of the suspension collected at T0 and at T24h are harvested and dropped on agarose of a petri dish SpiderMass analyses were performed in negative and positive modes. (Duplicates are presented for each conditions).

fluence was decreased to 0.8 J/cm^2 to allow for keeping it constant over the screened range. Three sample spots were deposited onto a glass slide to obtain significant results. The spectra acquisition were performed in positive resolution mode with a scan time of 0.5 sec.

Real-time analysis

For real time analysis, the built model was exported to a second software module called OMB Recognition. The parameters for this analysis were: a TIC threshold of 1E+4 count for irradiation detection, a normalized intensity threshold of 1E+6, and an outlier limit of 5 standard deviations. The software analyzes 1 scan per spectrum and has a 3s timeout waiting for good spectrum. The cross-validated models are exported from the OMB software and then loaded in the OMB Recognition software module before any real time piece of tissue analysis by the SpiderMass (18, 20). The real time acquired data are then directly interrogated, giving an immediate feedback using a color scale predefined from the specified classes.

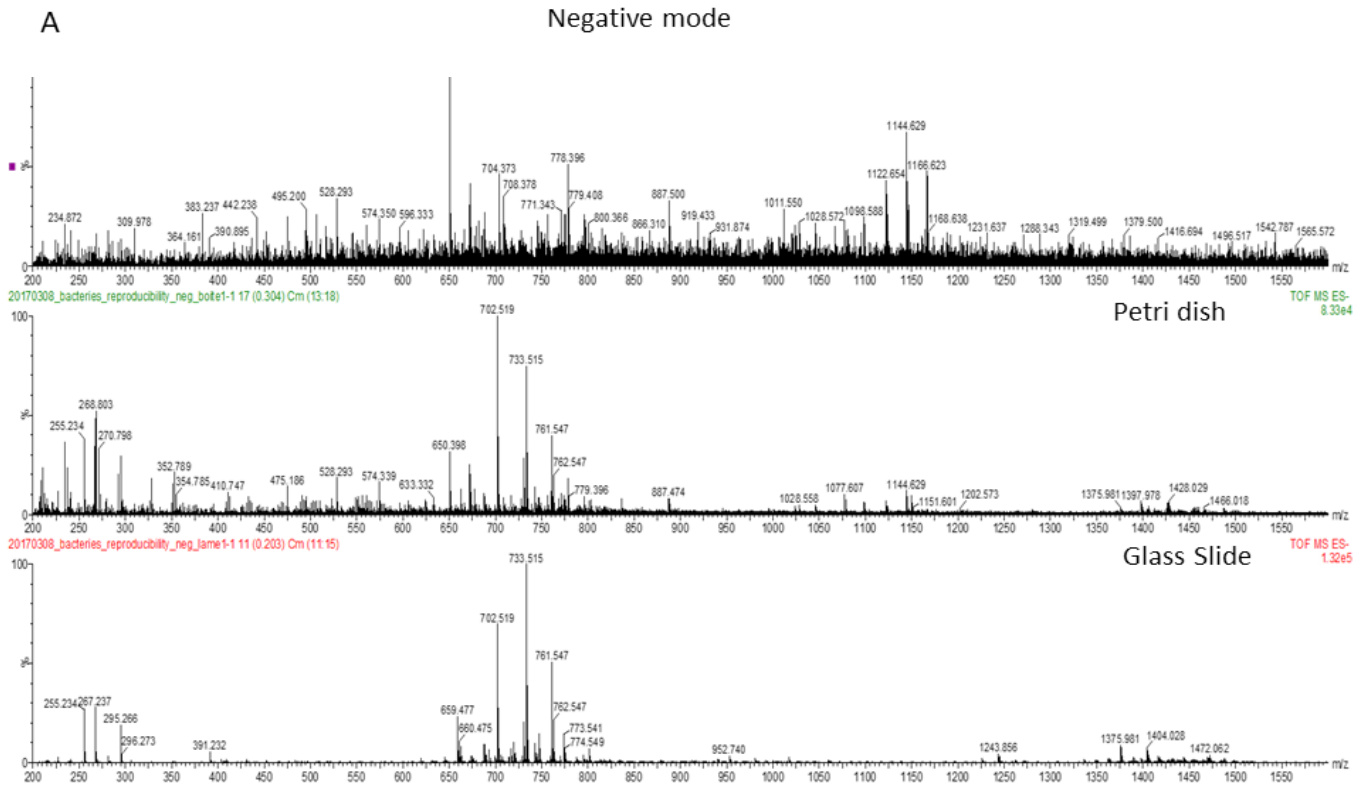
Lipid identification

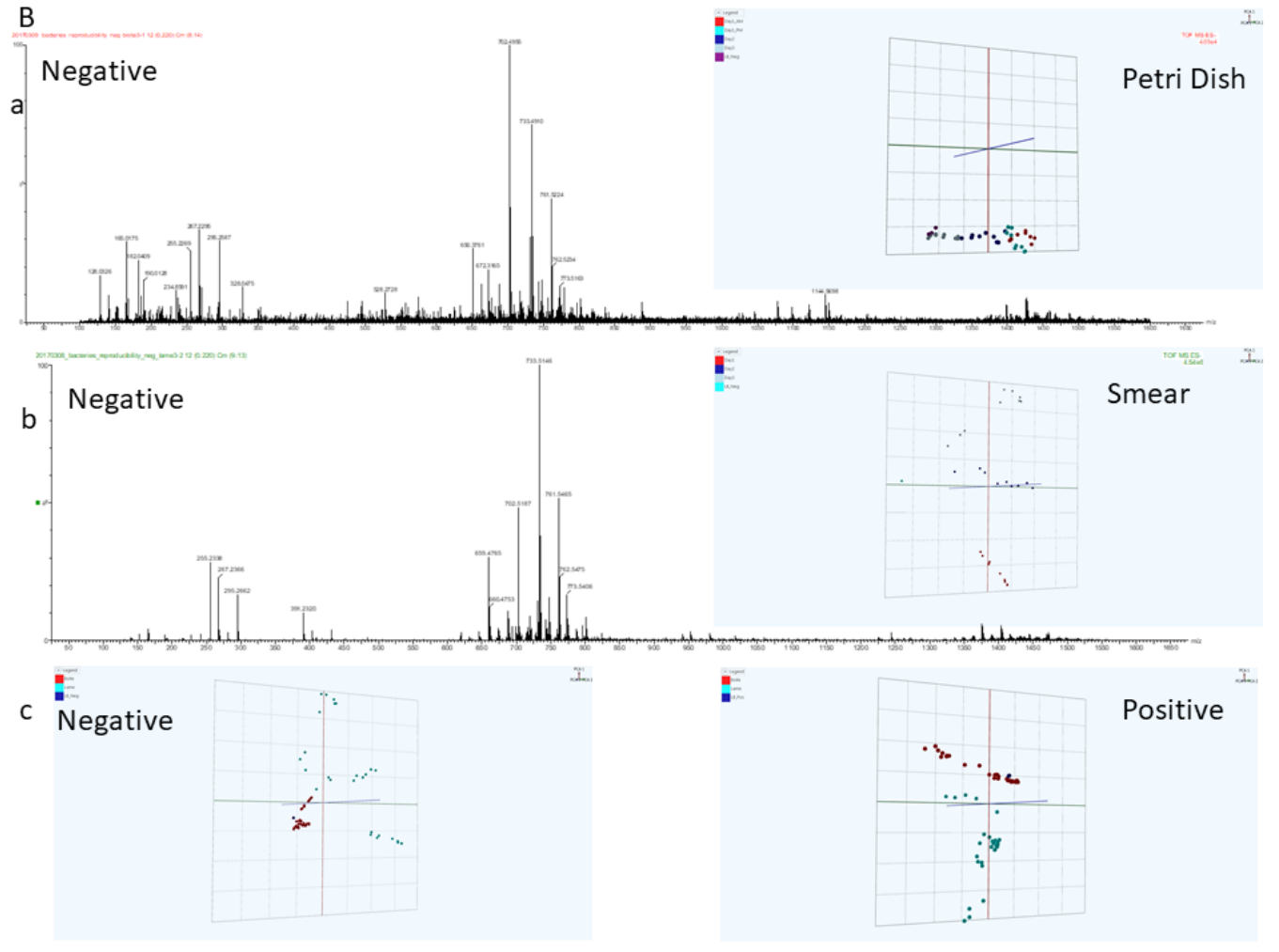
m/z intervals corresponding to loadings with the largest contribution to the explained variance observed in the different groups were selected for MS/MS-based identification. For these experiments, the settings were exactly the same as described in the Instrumentation section and published (18, 20). The identifications were performed directly on the tissue by doing a full scan first to verify the presence of the targeted masses. Then after switching to MS/MS mode, they were subjected to collision-induced dissociation (CID) in the transfer cell with respectively 20 and 35 V. The resulting spectra were annotated manually, and assignments were verified by interrogating the high accuracy mass measurements of the precursor ions using the LipidsMap database.

Results

Bacteria growth time courses

To assess the performance of SpiderMass for biotyping, we studied two different of sampling and analytical conditions instrument (**Fig. 1**). Time course analyses from T0 to T24 hours of *E. Coli* growths in liquid suspension were performed either directly from the agarose of the petri dish or from a smear spread on a glass slide. First, 10 µl of bacteria in suspension were harvested and dropped either on the agarose or smeared onto a glass slide and analyzed just after with SpiderMass in both negative and positive ion mode (**Fig. 2**). In negative of positive mode at T0, no specific m/z can be detected whatever the ion mode. Then bacteria were left growing for longer in time until 24H (T24) (**Fig. 2**). In time course, specific m/z are detected at T4h bacteria growth in negative mode i.e. 688.490, 719.491, 747.519 and 749.522. M/z 702.514 appears at T8 bacteria growth (**Supplementary Data 1**) and m/z at 733.515 increased at T24h bacteria growth (**Fig. 2**) In positive mode, first specific m/z (734.388, 748.390, and 764.370) appears at T6 bacteria growth. At T8 bacteria growth, these m/z increased and T24, ion at 748.790 is major (**Supplementary Data 1**). We then


A
Negative mode
Petri dish
Glass Slide


B
a
Negative
Petri Dish
b
Negative
Smear
c
Negative
Positive

C

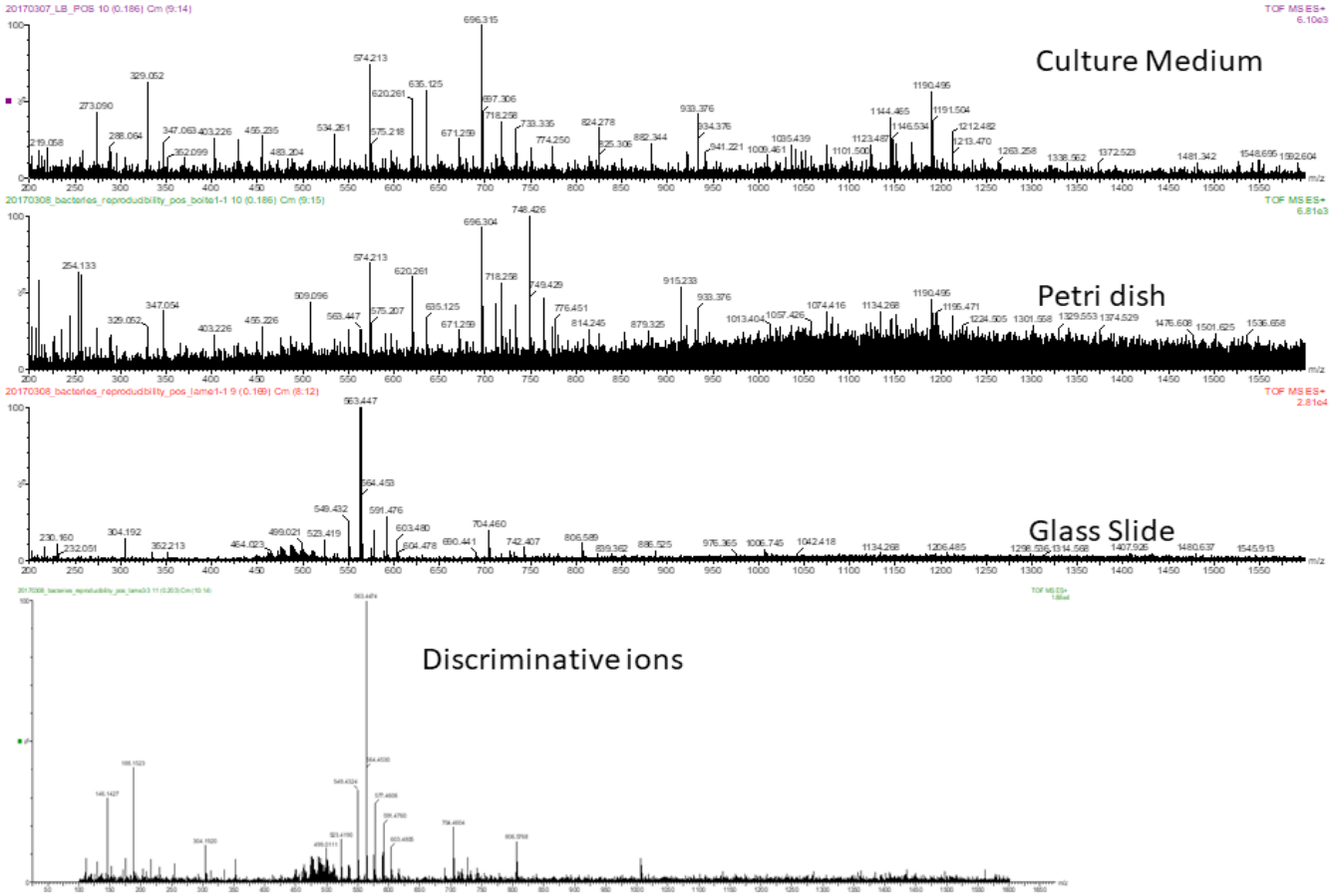


Figure 3. Comparison of SpiderMass spectra **A)** collected either from culture medium directly , agarose (petri dish) and on smears on glass slides for bacteria collected at T24, **B)** represent the PCA analyses in negative mode of the spectra collected from petri dish a); Smear b) and Comparison between direct medium, agarose and smear by PCA in negative and positive modes c). **C)** Highlights of specific common ions detected in positive mode ranged from 549.432 to 564.453 from the 3 methods.

compared tests performed on culture medium directly, agarose (petri dish) and on smears on glass slides for bacteria collected at T24 (**Fig. 3**). Results established that the smears performed on glass slides of the bacteria suspension gave the best results in term of intensity i.e. 1.32E5 vs 8.33E4 in agarose and 4.44E3 directly in medium, in negative mode (**Fig. 3A**). Some tiny differences are registered between glass slides and agarose, in fact ions m/e of 702.518 and 733.515 have not the same level of intensity in both conditions (**Fig. 3A**). PCA analyses of these two conditions in time course of bacteria growth (**Figs. 3Ba, 3Bb**) revealed that the discriminate ions between the two conditions are the same (**Figs. 3Ba, 3Bb**). Comparison between direct medium, agarose and smear by PCA confirmed that the 3 methods are different in negative and positive modes (**Fig. 3Bc**). Specific common ions are also detected in positive mode ranged from 549.432 to 564.453 (**Fig. 3C**). Based on these data, smears on glass slide was then selected instead direct analyses on agarose. Reproducibility experiments were conducted on 3 different slides containing smears of bacteria collected at T24. Experiments were realized on the same slides analyzed 3 times during 3 consecutive days in negative (**Fig. 4A**) and positive (**Fig. 4B**) modes. The same m/z were found in negative and positive modes whatever the slides and the time of the analysis reflecting the robustness of the technology.

Bacteria biotyping

The next step consisted to perform biotyping on different species of Gram negative (*E. coli D31, Pseudomonas aeruginosa);* Gram positive (St*aphylococcus aureus, Enterococcus faecalis)* and Yeast (*Candida Albicans*) (**Fig. 5).** None of them have the same ions detected. *C. alibicans* have characteristic ions centered on the most intense at 835.41. *E. Faecalis* presents 4 peak ranges (centered on m/z of 281.18, 747.40, 1403.89, 1545.89, and 1373.9). For *P. aeruginosa,* 5 peaks ranges are found (centered on m/z of 270.12, 716.41, 47.4, 1403.89, and 1603.92) and for *S. aureus*, a wide peak range centered on 735.39 is mostly discriminative. These results established the clear possibility to biotype human pathogens using the SpiderMass based on their metabolites and lipids profiles. In fact some of the m/z have been characterized by MS/MS using SpiderMass in MS/MS real time mode. In *E. coli*, ions at m/z of 688.49 corresponds to PE (16:0/16:1). The ones at m/z 688.35 to PE (16:0/cy17:0), m/z

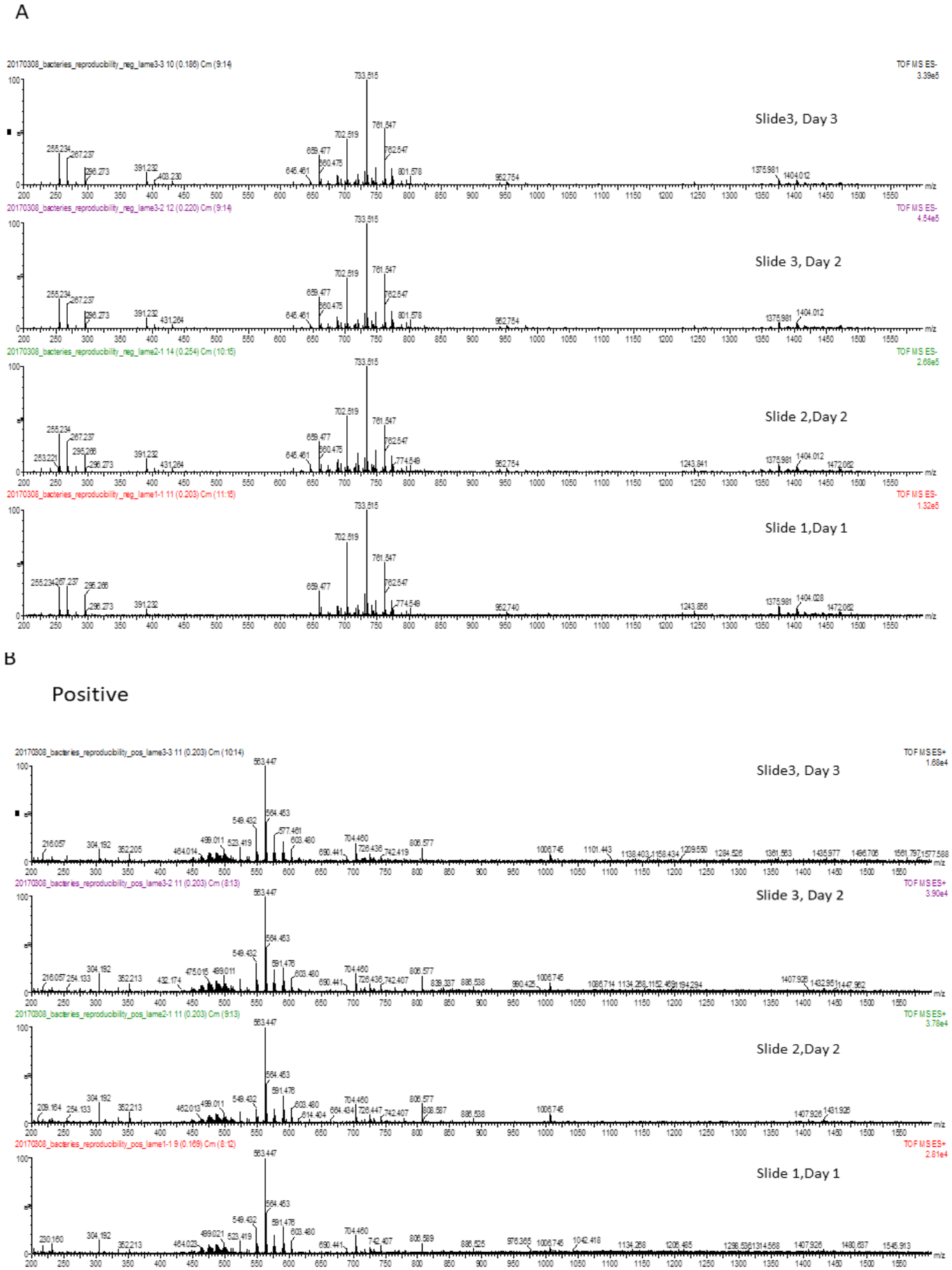


Figure 4. Reproducibility experiments were conducted on 3 different slides containing smears of bacteria collected at T24. Experiments were realized on the same slides analyzed 3 times during 3 consecutive days in negative **A)** and positive **B)** modes.

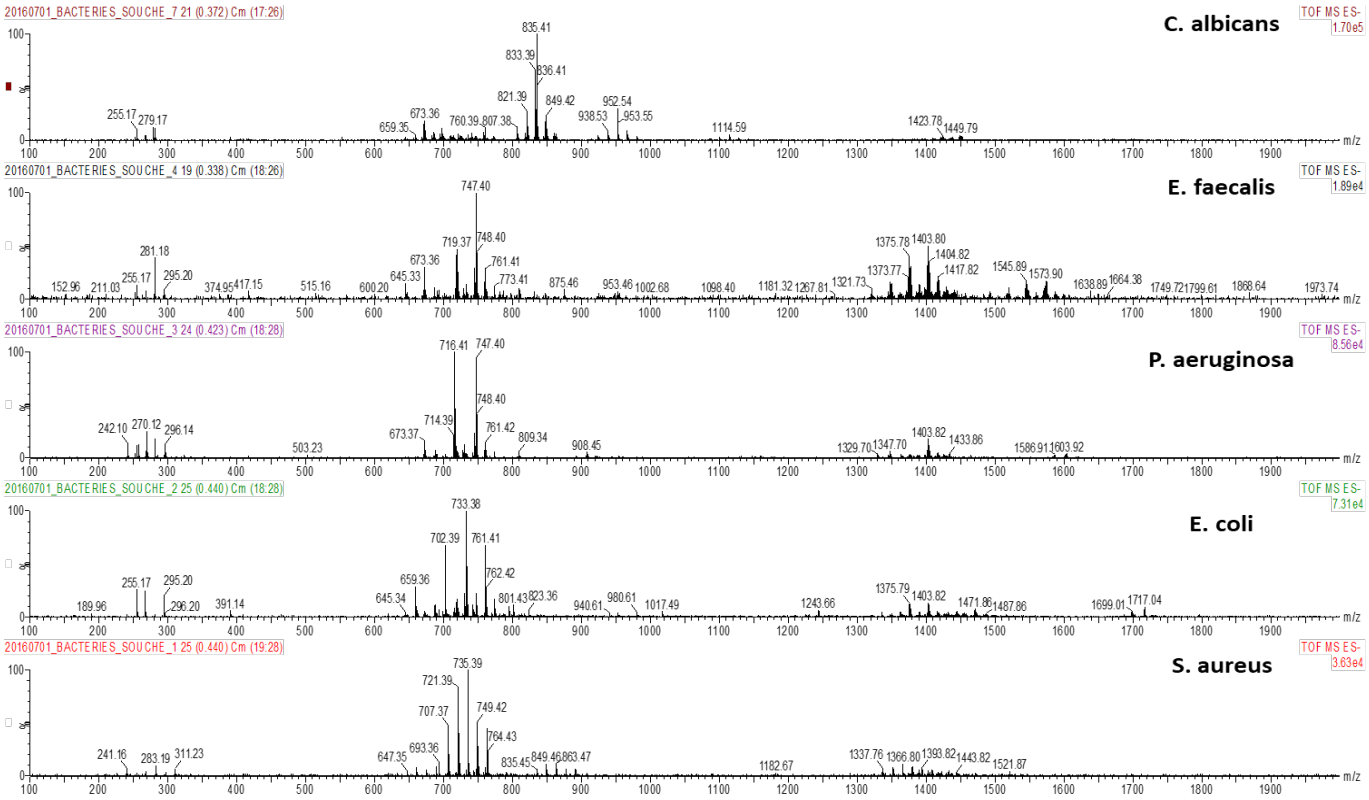


Figure 5. Biotyping studies in negative mode on smear of different species of pathogens *i.e.* Gram negative **(***E. coli D31, Pseudomonas aeruginosa);* Gram positive (St*aphylococcus aureus, Enterococcus faecalis)* and Yeast (*Candida Albicans*).

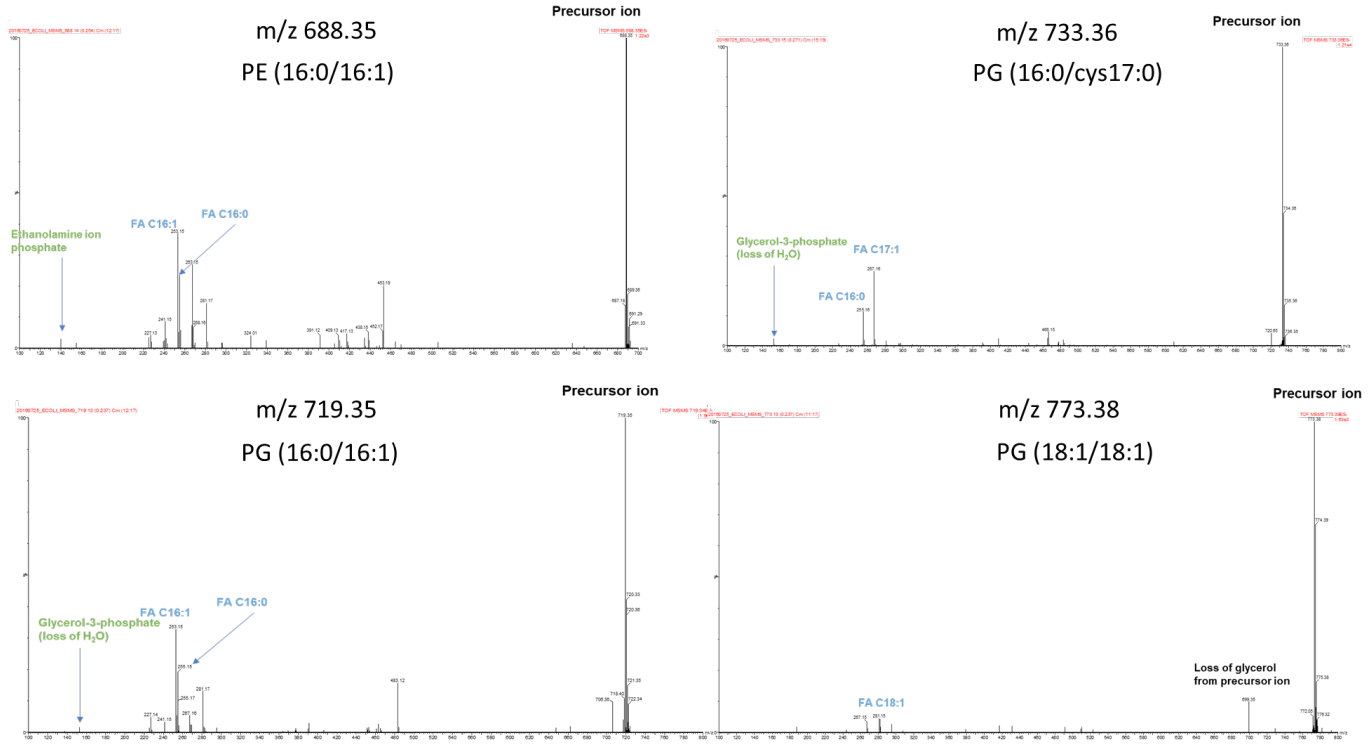


Figure 6. MS/MS analyses directly by SpiderMass in real time of selected ions from *E. Coli* corresponding to : m/z 688.35 to PE (16:0/cy17:0), m/z of m/z 719.35 to PG (16:0/16:1) and m/z 733.36 to PG (16:0/cys17:0), 773.38 to PG (18:1/18:1).

of m/z 719.35 to PG (16:0/16:1) and m/z 733.36 to PG (16:0/cys17:0), 773.38 to PG (18:1/18:1) (**Fig. 6**).

Discussion

We performed human pathogens bio-typing microorganisms using SpiderMass instrument in real time. In this context, we established that the most robust procedure based on smears of bacteria on glass slides. We then biotype human pathogens and observed the ability to distinguish Gram –; Gram + and yeast using SpiderMass technology based on their metabolites and lipids signatures. It is interesting to note, that specific bacteria signature can be detect at 4 hours growth in negative mode whereas other bio-typing technologies need at least 24hours culture growth and protein extraction procedures. Using SpiderMass same types of information is now given from 4 hours culture without any treatment. Taken together, we established that SpiderMass can be a useful instrument for bacteria biotyping in the clinical context and open the door of on tissue bacteria biotyping as well as in liquid for detecting Multi-resistant bacteria.

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Author contributions

IF, MS have written the paper. BF have done the experiments. MS, IF have got financial support to the project and corrected the manuscript. All authors have reviewed the manuscript.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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